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STUDIES ON PLANT GUMS OF THE ACACIA GROUP

by

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Thesis presented for the degree of Doctor of Philosophy

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TO MY PARENTS

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CHAPTER I

GENERAL INTRODUCTION

Several hundred species of the genus Acacia, subfamily Mimosoideaea and family Leguminosae, are known botanically. Exudation of nodules of gummy composition is a phenomenon exhibited by trees belonging to the various species of this genus. The nodules are composed predominantly of carbohydrate material in the form of a salt of an acidic polysaccharide.

In spite of the fact that a considerable amount is known about the primary chemical structural features of the polysaccharides exuded by some Acacia species, the reasons for gum formation are still obscure. Although it seems improbable that a mere knowledge of structural features alone will lead to a satisfactory explanation of the reasons for, and of the biosynthetic mechanisms involved in, gum formation, some understanding of primary chemical structure is undoubtedly a prerequisite to any biochemical investigation.

From chemical and physico-chemical aspects, Acacia senegal (syn. verek) gum, frequently referred to as gum arabic in the literature, has been the most extensively studied of the Acacia gums. Chapter III of this thesis deals with a re-investigation of the structural features of this gum. In Chapter IV an account of a detailed examination of Acacia arabica gum is given, and the main structural features of this gum are compared

with those of A.senegal gum. An attempt is made to discuss their primary chemical features in relation to their average molecular sizes and possible molecular shapes. A short summary is presented in Chapter V, and the general methods applied to the elucidation of problems in molecular structure are outlined in Chapter II.

It is hoped that the choice of techniques described in this thesis to study the molecular structures of Acacia gums reflects the need for a more diversified and a less specialised approach to experimental design in gum chemistry.

CHAPTER II

MATERIALS AND METHODS

2.1) Materials.

The nodules of A.senegal (L.) Willd. (syn. verek) gum were collected by (the late) Mr. M.P. Vidal-Hall, formerly Gum Research Officer, Republic of the Sudan, at Qala en Nahal, Kassala Province, as the first collection of the 1960 gum season. The nodules of A.arabica (Lam.) Willd. gum were collected from small trees at Baruki Rahad, Kardofan Province, in March, 1961.

2.2) General Methods.

Weighings.- By adjustment of tares, all accurate weighings were made within the range of the graticule scale (range, 0-30 mg.) on an aperiodic direct-reading balance having an accuracy of $\pm$ 10 μg.

Dialyses of polysaccharides were carried out in cellophane tubing (Kalle Aktiengesellschaft, Wiesbaden) against running tap-water unless otherwise stated.

Electrodialyses of polysaccharides were carried out in a three-compartment cell fitted with cellophane membranes; constant stirring of the gum solution in the middle compartment guarded against the occurrence of electrodecantation (Morris & Morris, 1963).

Evaporations were carried out on a rotary evaporator at temperatures ca. 35°.

2.3) Physical Methods.

Optical rotations were observed for aqueous solutions, unless otherwise stated, at 20 ± 2°C. Values for concentrations used in calculations of specific optical rotations were corrected for moisture contents.

Infrared measurements were made on a Hilger H 800 double-beam spectrometer, fitted with a sodium chloride prism.

X-Ray diffraction patterns were recorded on a Philips BW 1051 Diffractometer (Cu K_α radiation).

X-Ray powder photographs were taken with a 9 centimeter Unicam powder camera (Cu K_α radiation).

(I wish to thank Dr. H.A. Long for his instruction and assistance in the use of these X-ray techniques).

Viscosity determinations were carried out in M-sodium chloride solution in a suspended-level dilution viscometer at 25.00 ± 0.01°C. Solutions were filtered carefully before additions were made to the viscometer. Flow-times were measured to within 0.1 secs. by means of a stop-watch. The viscometer had a flow-time of 189.9 secs. for M-sodium chloride solution. The technique of isionic dilution was used; gum solution (10 ml.) was placed in the viscometer and the flow-time measured. Flow-times were also obtained for successive dilutions with M-sodium chloride solution (two additions of 5 ml. each, followed by one of 20 ml.). Since preliminary experiments indicated that any loss of gum from M-sodium chloride solution on filtering was negligible, concentration values were estimated from a knowledge of the weight (dry weight basis) of gum dissolved in a known volume.

The viscosity number, η_{sp}/c, of the gum solution is given by

$$\frac{\eta}{c}sp = \frac{\eta - \eta_o}{c\eta_o}$$

where η_{sp} is the specific viscosity, η_o is the viscosity coefficient of the M-sodium chloride solution, and η is the viscosity coefficient of the gum solution of concentration, c (in g./ml.). Since the viscometer was designed (cf. Banks & Greenwood, 1963) to minimise any error incurred by neglecting kinetic energy corrections, it may be assumed that

$$\frac{\eta}{c}sp = \frac{\rho t - \rho_o t_o}{c\rho_o t_o}$$

where ρ_o and ρ are the densities of the M-sodium chloride solution and gum solution respectively, and t_o and t are the flow-times for a fixed volume of M-sodium chloride solution and gum solution respectively. At low concentrations of gum in solution, ρ may be assumed equal to ρ_o, and hence

$$\frac{\eta}{c}sp = \frac{t - t_o}{ct_o}$$

Since η_{sp}/c is approximately a linear function of c, extrapolation of $(t - t_o)/ct_o$ to c = 0 gives the limiting viscosity number, $[\eta]$, in units of reciprocal concentration (i.e. in ml./g.).

Light-scattering measurements were carried out at 25.00 ± 0.02°C using a photometer based on the design of McIntyre & Doderer (1959) and constructed in the Procter Department, University of Leeds for

Dr. G. Stainsby. Details of this instrument and its calibration have been described by Hey (1965). Unpolarised blue light (4358 Å) was selected from a mercury lamp spectrum by a combined 47B and 2E Wratten filter.

Known weights of gum were dissolved in known volumes of M-sodium chloride solution; solutions were clarified by ultrafiltration through 5 μ or 1.2 μ "Millipore" filters using a stainless steel filter-holder attached to a 50 ml. syringe. Filtrates were transferred directly into a previously calibrated cylindrical light-scattering cell (approximate minimum capacity, 70 ml.); concentrations of gum solutions were assumed to be the same as those prior to ultrafiltration (cf. Rahman, 1966).

When the photometer is in use, one galvanometer, G_1, monitors the primary beam, while a second galvanometer, G_2, monitors light scattered at any angle between 0° and 160° after suitable attenuation. The intensity of scatter for a gum solution is given by

$$G'_\theta = \frac{(G_2/G_1)_\theta}{(G_2/G_1)_0}$$

where $(G_2/G_1)_\theta$ is the ratio of the galvanometer readings at θ°, and $(G_2/G_1)_0$ is the ratio at 0°. An intensity of scatter, G''_θ, was also obtained from M-sodium chloride solution. Thus, the intensity of scatter, $\Delta G'_\theta$, of the gum molecules in solution is given by

$$\Delta G'_\theta = G'_\theta - G''_\theta$$

The reduced intensity of scatter, ΔG_θ, is then expressed by

$$\Delta G_\theta = \Delta G'_\theta [\sin \theta/(1 + \cos^2\theta)]$$

where $\sin \theta/(1 + \cos^2\theta)$ is a correction term, obtainable from Tables (Stacey, 1956), for volume viewed and partial polarization, which both vary with θ.

For small solute particles, the intensity of scattered light is symmetrical about a position normal to the incident beam. If the incident light is unpolarised, the molecular weight, M, of the solute molecule in an ideal solution is related to its concentration, c, by the equation

$$\frac{Kc}{R_\theta} = \frac{1}{M} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where

$$R_\theta = \frac{I_\theta}{I_0} \cdot \frac{r^2}{(1 + \cos^2\theta)}$$

[I_θ = intensity of scattered light at angle θ

I_0 = intensity of incident light

r = distance from solute molecule to detector]

and

$$K = \frac{2\pi^2 n_o (dn/dc)^2}{N\lambda^4}$$

[n = refractive index of solution

n_o = refractive index of solvent

N = Avogadro's number

λ = wavelength of incident light in solution]

For non-ideal solutions, solute-solvent and solute-solute

interactions, which are concentration dependent, must be considered. The equation

$$\frac{Kc}{R_\theta} = \frac{1}{M}(1 + 2BMc + \ldots) \quad \ldots\ldots\ldots (2)$$

where B is the second virial coefficient, takes such interactions into account.

Since the macromolecules in a gum solution exhibit a wide range of molecular weights,

$$R_\theta(1 + 2B\sum_{i=1}^{\infty} c_i M_i + \ldots) = K\sum_{i=1}^{\infty} c_i M_i \quad \ldots\ldots (3)$$

where c_i is the concentration of the ith molecules by weight, and M_i is their molecular weight. Since

$$c = \sum_{i=1}^{\infty} c_i \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

it follows that

$$R_\theta\left\{1 + \frac{2Bc\sum_{i=1}^{\infty} c_i M_i}{\sum_{i=1}^{\infty} c_i} + \ldots\right\} = \frac{Kc\sum_{i=1}^{\infty} c_i M_i}{\sum_{i=1}^{\infty} c_i} \quad \ldots\ldots (5)$$

By definition, the weight-average molecular-weight, $\bar{M}_w$, is given by

$$\bar{M}_w = \frac{\sum_{i=1}^{\infty} c_i M_i}{\sum_{i=1}^{\infty} c_i} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (6)$$

and so equation (5) becomes

$$\frac{Kc}{R_\theta} = \frac{1}{\bar{M}_w}(1 + 2B\bar{M}_w c + \ldots) \quad \ldots\ldots\ldots (7)$$

For macromolecules, destructive interference between light scattered from different parts of the molecules leads to a loss

in the scatter intensity. To compensate for this loss, a particle scattering factor, P_θ, which is defined as the ratio of the scattered intensity at angle θ to the scattered intensity at angle θ in the absence of interference, is introduced into equation (7):-

$$\frac{Kc}{R_\theta} = \frac{1}{\bar{M}_w P_\theta}(1 + 2B\bar{M}_w c + \ldots) \quad \ldots\ldots\ldots \quad (8)$$

Values for $\bar{M}_w$ may now be calculated; an extrapolation method introduced by Zimm (1948) may be used without making any assumptions about the shape of the macromolecule. At $\theta = 0^o$, $P_0 = 1$, and so

$$\frac{Kc}{R_0} = \frac{1}{\bar{M}_w}(1 + 2B\bar{M}_w c + \ldots) \quad \ldots\ldots\ldots \quad (9)$$

In this method, Kc/R_θ is plotted against $\sin^2\theta/2 + kc$ where k is an arbitrarily chosen constant to give a good spread of the experimental data on a grid-like graph. The intercept, $(Kc/R_\theta)_{\theta = 0;\ c = 0}$, obtained on extrapolation, is equal to the reciprocal of $\bar{M}_w$. In the present investigation a full Zimm plot could not be used because of the unsystematic variation of $\bar{M}_w$ with c (*cf.* Anderson, Hirst, Rahman & Stainsby, 1966). To a first approximation, it is assumed that Kc/R_0 is independent of c, *i.e.* the virial coefficients are zero. This assumption appears to be reasonable, since the use of M-sodium chloride solution as solvent should minimise interactions between neighbouring gum molecules. Thus, equation (9) reduces to

$$\frac{Kc}{R_0} = \frac{1}{\overline{M}_w} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (10)$$

Since R_0 is proportional to the reduced intensity of scatter, ΔG_0, it follows that

$$R_0 = k\Delta G_0 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (11)$$

where k is a combined calibration and cell constant, which was equal to 3.74×10^{-5} for the particular instrument and cell that were used. From equations (10) and (11), it follows that

$$\overline{M}_w = \frac{k}{Kc\Delta G_0^{-1}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (12)$$

The reciprocal reduced intensity of scatter, ΔG_θ^{-1}, is plotted against $\sin^2\theta/2$ [obtainable from Tables (Stacey, 1956)]. Extrapolation of the linear portion of this graph to $\theta = 0^o$ gives a value for ΔG_0^{-1}. The downward curvature of these graphs is thought (Stainsby, 1966; Anderson et al., 1966) to be caused by scatter from dust particles suspended in solution. Clarification of aqueous solutions is difficult to achieve (Stacey, 1956; Banks & Greenwood, 1963); although this scatter from residual impurities is indeterminate (Anderson, et al., 1966), it will undoubtedly lead to high values for $\overline{M}_w$. Concentration values were expressed in units of g./ml. The value of 3.77×10^{-7} for K, obtained by Veis & Eggenberger (1954), and confirmed by Rahman (1966) for gum arabic dissolved in M-sodium chloride solution, was used to calculate values for $\overline{M}_w$.

2.4) Chemical Methods.

Small-scale polysaccharide hydrolyses.- Polysaccharides were hydrolysed with N-sulphuric acid for 7 hours on a boiling-water bath; these conditions do not cause any extensive hydrolysis of the uronosyl linkages in Acacia gums; this was taken into account when determining proportions of galactose. Hydrolysate solutions were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated on a rotary evaporator.

Small-scale polysaccharide methylations.-

(a) Haworth (1915).- Methylations were carried out in an atmosphere of nitrogen at room temperature. Dimethyl sulphate (2 ml.) and sodium hydroxide solution [2 ml., 30% (w/v)] were added dropwise with stirring to the polysaccharide (100 - 500 mg.) in water (10 ml.) over a period of 1 hour. Acetone (5 ml.) was added to the reaction mixture, and further additions of dimethyl sulphate (12 ml.) and sodium hydroxide solution (17 ml.) were made over periods of 3 hours for each addition; five or six such additions were made. After stirring for 12 hours, the reaction mixture was heated at 60° for 30 mins., while nitrogen was bubbled vigorously through the solution. After cooling, the reaction mixture was neutralised with 5N-sulphuric acid and made slightly acid (pH, 4.0); a white precipitate usually formed at this stage. The methylated product was extracted into chloroform (3 x 60 ml. extractions) and the extract shaken with saturated sodium chloride solution (ca. 100

ml.). The chloroform layer was separated, dried over anhydrous sodium sulphate and concentrated on a rotary evaporator. The concentrated chloroform solution was poured into light petroleum (b.p. 60° - 80°; ca. 200 ml.) with stirring; the methylated product was isolated as a white amorphous powder.

(b) Purdie & Irvine (1903).- The partially methylated product (100-400 mg.) was dissolved in methanol (5 ml.) and methyl iodide (10 ml.) added. Silver oxide (1 g.) was added in four batches of ca. 250 mg. every 1.5 hours; the mixture was refluxed for 6 hours in a dry flask fitted with a water condenser and a calcium chloride tube. The mixture was cooled, filtered through sintered glass, and the residue extracted five times with hot chloroform. The combined filtrate and extracts were concentrated to a small volume. The concentrated chloroform solution was poured into light petroleum (b.p. 60° - 80°; ca. 200 ml.) with stirring; the methylated product was isolated as a white amorphous powder.

(c) Kuhn, Trischmann & Löw (1955).- The partially methylated product (100-400 mg.) was dissolved in N,N-dimethylformamide (5 ml.) and methyl iodide (5 ml.). Silver oxide (2.5 g.) was added and the mixture was shaken at room temperature in the dark for 18 hours. The mixture was filtered and the residue extracted with chloroform. The combined filtrate and extracts were reduced to a syrup on a rotary evaporator; the syrup was taken up in a small volume of chloroform; the concentrated chloroform solution was poured into light petroleum (b.p. 60° - 80°;

ca. 200 ml.) with stirring; the methylated product was isolated as a white amorphous powder.

Small-scale oligosaccharide methylations.- (Kuhn et al., 1955; Perila & Bishop, 1961).- The oligosaccharide (0.5-2.0 mg.) was shaken with methyl iodide (0.2 ml.), N,N-dimethylformamide (0.2 ml.) and silver oxide (200 mg.) at room temperature in the dark for 18 hours. The mixture was filtered and the residue washed with chloroform; the combined filtrate and washings were reduced to a syrup on a rotary evaporator.

Methanolyses.- Unless otherwise stated, methanolyses were carried out under reflux for 6 hours with methanolic 5% hydrogen chloride. Solutions were neutralised with silver carbonate and filtered; the residue was washed with methanol and the combined filtrate and washings were reduced to a syrup on a rotary evaporator.

Demethylations.- (Hough, Jones & Wadman, 1950) were carried out by heating the O-methyl sugar (ca. 5 mg.) with hydriodic acid (1 ml.) in a sealed tube for 5 mins. at 100°C. The solution was diluted with water and neutralised with silver carbonate; silver ions were removed by bubbling hydrogen sulphide through the solution; after filtration and washing, the combined filtrate and washings were taken to dryness on a rotary evaporator; the syrup was examined by paper chromatography.

Periodate oxidations of polysaccharides.- Periodate oxidations were carried out at room temperature unless otherwise stated.

(a) Consumption of periodate.- The amount of periodate consumed by a polysaccharide was estimated by back-titration of excess of periodate. Saturated potassium iodide was added in excess to the periodate solution; the iodine released was titrated, after addition of sodium bicarbonate (ca. 200 mg.), with standard sodium arsenite solution (ca. 0.1N) using "Thyodene" as indicator (Belcher, Dryhurst & Macdonald, 1965).

(b) Formic acid released was estimated titrimetrically (Halsall, Hirst & Jones, 1947). Excess of periodate was destroyed by addition of ethylene glycol; formic acid was titrated with standard sodium hydroxide solution (ca. 0.1N) using methyl red as indicator.

(c) Formaldehyde released was estimated colorimetrically with chromotropic acid by the procedure outlined by Annan, Hirst & Manners (1965).

Periodate oxidations of O-methyl sugars.- (Lemieux & Bauer, 1953).- The O-methyl sugar (1-2 mg.) was treated with 0.05M-sodium metaperiodate solution (0.2 ml.) at 0°C for 1 hour. Excess of periodate was destroyed by addition of ethylene glycol (1 drop); after 5 mins., the solution was made alkaline to phenolphthalein by addition of 0.5N-sodium hydroxide solution. The solution was reduced to dryness and extracted with acetone; the acetone extract was taken to a syrup, which was examined by paper chromatography.

2.5) Chromatographic Separations.

Molecular-sieve chromatography.- This was carried out on columns (50 x 4.8 cm.) of "Bio-Gel P300" (Bio-Rad Laboratories, Richmond, California) using M-sodium chloride solution as eluant (Anderson, Dea, Rahman & Stoddart, 1965). Thymol (10 mg.) was added to the M-sodium chloride solution (5 l.) as a bacteriostatic agent. To prevent deformation by "wall-effects," columns were pretreated with 1% dichlorodimethylsilane in benzene at 60°C. After oven-drying, columns were packed with gel which had been allowed to swell in M-sodium chloride solution for 2 days; small particles of gel were removed by repeated decantation. A thin layer of glass beads at the bottom of the column was used to support the gel; care was taken to keep the "dead-space" to a minimum. The gel slurry was added continuously to the column from a separating funnel (6 in. diam.); the gel slurry in the separating funnel was stirred while excess of M-sodium chloride solution was allowed to percolate through the growing gel bed by regulating the flow of liquid from the tap at the bottom of the column to a rate of approximately 30 ml. per hour. In order to stabilise the soft top surface of the "P300" gel, 1 cm. layers of "Bio-Gel P200" and "Bio-Gel P10" were applied successively to the column. Eluant was allowed to flow through the columns for 2 days before they were used. Each column was checked for evenness of packing by observing the passage through it of a band of "blue-dextran" (Pharmacia Ltd., Uppsala). Column calibration was achieved with dextran fractions (Pharmacia Ltd.,

Uppsala) of known number-average molecular-weight, $\bar{M}_n$. Columns were eluted continuously with M-sodium chloride solution when not in use.

Polysaccharide (ca. 10 mg.), dissolved in 1.5M-sodium chloride solution (1 ml.), was applied to the column by careful layering beneath the M-sodium chloride solution. Fractions, collected from a 2 ml. siphon by an automatic collector, were screened directly using the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The optical density was read on a Unicam SP 1300 spectrophotometer using filter 2. Collection of fractions was commenced as soon as the polysaccharide had been applied to the top of the column. Elution volumes, V_e, were estimated to the nearest 1 ml. from an elution diagram, by extrapolating both sides of the solute peak to an apex.

Ion-exchange chromatography on diethylaminoethylcellulose (DEAE-cellulose).- (Jermyn, 1962).- DEAE-cellulose powder (Whatman DE 32, microgranular form, 10 g.) was treated with 0.5N-hydrochloric acid (250 ml.) for 30 mins. After gentle filtration and washing until the effluent pH was ca. 4, the exchanger was treated with 0.5N-sodium hydroxide solution (200 ml.) for 30 mins. After further filtration and washing until the effluent was neutral, columns (30 x 1.5 cm.) were packed by the technique described in detail for "Bio-Gel P300" columns. Columns were equilibrated with 0.02M-acetate buffer (pH, 4.1). Samples (50 mg.) of gum were dissolved in buffer (2 ml.) and washed into the column with excess of buffer; elution of the

acidic polysaccharide was performed by application of a sodium chloride concentration gradient (0-0.3M) in 0.02M-acetate buffer (pH, 4.1). Fractions, collected from a 15 ml. siphon by an automatic collector, were sampled (1 ml.); the samples were screened by the phenol-sulphuric acid method (Dubois et al., 1956). The optical density was read on a Unicam SP 1300 spectrophotometer using filter 2.

Cellulose column chromatography.- Cellulose columns were packed with dry Whatman Cellulose Powder CF 1 and washed with water; they were then washed with the solvent system to be used for separating the sugars. After excess of the solvent had been removed from the top of the column, sugar mixtures were added in minimum amounts of eluant and allowed to soak into the cellulose at the top of the column. Sugars were eluted from the column using the given eluants; fractions were collected from a 20 ml. siphon on an automatic collector; a sample (ca. 5 ml.) from every tenth tube was reduced to dryness and examined for sugars by paper chromatography. Tubes containing the same sugars were combined; solutions were reduced to dryness; residues were extracted with warm acetone:water (1:1 v/v), treated with charcoal, and filtered; filtrates were reduced to dryness, dried in a vacuum desiccator, and weighed.

Paper chromatography of sugars was carried out on Whatman No.1 papers using the following solvent systems (by vol.):-

(a) benzene-butan-1-ol-pyridine-water (1:5:3:3, upper layer)

(b) ethyl acetate-acetic acid-formic acid-water (18:3:1:4)

(c) butan-1-ol-ethanol-water (4:1:5, upper layer)

(d) butan-1-ol-acetic acid-water (4:1:5, upper layer)

(e) ethyl acetate-pyridine-water (10:4:3)

(f) butanone-water-ammonia (d, 0.880) (200:17:1)

(g) benzene-ethanol-water (169:47:15, upper layer)

(h) butanone-acetic acid-water (9:1:1, satd. with boric acid).

The following developers were used for identification of sugars:-

(1) Reducing sugars were detected by spraying with a saturated solution [water:ethanol, 1:1 (v/v)] of aniline oxalate and heating at 120°C for ca. 5 mins.

(2) A 3% (w/v) solution of p-anisidine hydrochloride in butan-1-ol was used to detect reducing sugars; after spraying, chromatograms were heated at 120°C for ca. 5 mins.

(3) Reducing sugars and sugar alcohols were detected by dipping chromatograms in silver nitrate reagent [saturated aqueous silver nitrate solution (1 ml.) added to acetone (100 ml.) and sufficient water added to redissolve the precipitate which formed], drying and spraying with aqueous ethanolic N-sodium hydroxide solution [water:ethanol, 1:9 (v/v)]. Chromatograms were preserved by treating with a 10% (w/v) sodium thiosulphate solution and washing with water.

(4) Sugar alcohols were detected by spraying with a mixture of 4 parts of 2% (w/v) sodium metaperiodate solution to 1 part (by volume) of 1% (w/v) potassium permanganate in 2% (w/v) sodium carbonate solution.

R_F values of sugars refer to distances moved relative to that

of the solvent front.

R_{gal} values of sugars refer to distances moved relative to that of galactose.

R_G values of O-methyl sugars refer to distances moved relative to that of 2,3,4,6-tetra-O-methyl-D-glucose.

Thin-layer chromatography of sugars was carried out on "Chromagram" sheets (Kodak Ltd., Kirkby, Liverpool) of polycarbonate or silica gel (Anderson & Stoddart, 1966a), using the following solvent systems (by vol.):-

(i) propan-1-ol-ethyl acetate-water (10:3:1) with polycarbonate

(j) butan-1-ol-acetone-water (4:5:1) with silica gel

(k) butanone-acetic acid-water (3:1:1) with silica gel.

Aniline oxalate or periodate permanganate sprays were used as developers.

Gas-liquid partition chromatography (Chromatograph Type S3A, fitted with flame ionisation detectors, supplied by Gas Chromatography Ltd.) of mixtures of O-methyl sugars (Bishop & Cooper, 1960; Aspinall, 1963) was carried out at nitrogen flow-rates of ca. 100 ml./min. on columns of:-

(i) 15% by weight of butan-1,4-diol succinate polyester on 60-70 mesh Celite (5 ft. x 1/4 in.) at 175°C.

(ii) 15% by weight of ethylene glycol adipate polyester on 60-70 mesh Celite (3 ft. x 1/4 in.) at 160°C.

Retention times, T, are quoted relative to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside as standard.

Gas-liquid partition chromatography of mixtures of

O-trimethylsilyl sugars (Hedgley & Overend, 1960; Sweeley, Bentley, Makita & Wells, 1963; Ludlow, Harris & Wolf, 1966) was carried out at nitrogen flow-rates of ca. 100 ml./min. on a column of:-

(iii) 3% by weight of SE 53 on 60-70 mesh Celite (5 ft. x 1/4 in.) at 130°C.

Retention times, T', are quoted relative to fully trimethylsilylated-α-D-glucopyranoside as standard.

2.6) Zone electrophoretic separations.

Separations were carried out on the following instruments:-

1. A Shandon Universal Electrophoresis Apparatus, Mark II [after Kohn (1958)] fed by a Vokam power unit, capable of providing constant voltage (max. 400 volts) or constant current, was used for strips up to 18 cm. in length.

2. A ridgepole tank (Durrum, 1950) was used with the above power unit for strips 36 cm. in length.

3. A Shandon Continuous Electrophoresis Apparatus was used with a high voltage power pack (max. 1000 volts).

(I wish to thank Dr. H. T. Macpherson for the use of his continuous electrophoresis apparatus).

Zone electrophoresis of polysaccharides.-

(a) Glass-fibre.- (Lewis & Smith, 1957).- Experiments were carried out on strips (18 x 5 cm.) of Whatman GF/A paper using 2N-sodium hydroxide solution as electrolyte; strips were soaked in electrolyte, pressed gently between sheets of filter paper and placed in the electrophoresis apparatus. Polysaccharide (ca.

1 mg.) in 2N-sodium hydroxide solution (ca. 30 µl.) was applied as a band across the strips near the cathode end. A potential of about 300 volts across a 16 cm. length was applied via wicks of double thickness of Whatman No.3MM paper for 24 hours; glass-fibre strips were dried at 100°C and sprayed (Briggs, Garner & Smith, 1956) with 0.5% (w/v) potassium permanganate in N-sodium hydroxide solution.

(b) Paper.- Experiments were carried out in the ridgepole tank on strips (18 x 5 cm.) of Whatman No.1 paper, using 0.1M-ammonium carbonate (pH, 8.9) as buffer. Strips were prepared for runs as described for glass-fibre papers; electrophoresis was carried out at a field strength of 5 volts/cm. for 24 hours.

(c)Cellulose acetate.- Experiments were carried out on strips (18 x 5 cm.) of cellulose acetate film (obtainable from Schleicher and Schüll, Dassel) using either 0.1M-ammonium carbonate buffer (pH, 8.9) or 0.1M-acetate buffer (pH, 4.7) as electrolytes. The procedure, recommended by Sargent (1965), was followed for preparation of cellulose acetate film; the strip was dropped into a shallow tray containing buffer so that the lower surface only was in contact with buffer; after buffer had soaked into the cellulose acetate, the strip was immersed; the strip was then blotted lightly between sheets of filter paper to remove excess of buffer and placed in the electrophoresis apparatus. Electrophoresis was carried out at a field strength of between 15-20 volts/cm. for 2-4 hours.

The following developers were used for identification of

polysaccharides on paper and cellulose acetate strips:-

1. Periodate-rosaniline hydrochloride.- (Conacher & Rees, 1966).

Solution a. Sodium metaperiodate (2 g.) was added to a mixture of water (30 ml.) and ethanol (200 ml.). This solution was prepared each day.

Solution b. Rosaniline hydrochloride (2 g.) was dissolved in water (250 ml.) and sulphur dioxide was bubbled gently through the solution for 10 mins. Activated charcoal (5 g.) was added; the solution was allowed to stand for 20 mins. before filtering. The reagent could be regenerated by passage of sulphur dioxide, followed by treatment with activated charcoal and filtration.

The following staining procedure was adopted:-

(i) Strips were immersed in ethanol for 20 mins.

(ii) Strips were immersed in solution a for 10 mins.

(iii) Strips were washed with ethanol.

(iv) Strips were immersed in solution b until staining was complete (5-10 mins.).

(v) Strips were washed twice in a solution of potassium bisulphite (1 g.) and concentrated hydrochloric acid (2 ml.) in water (300 ml.).

(vi) Strips were washed in ethanol and dried between sheets of glass.

Polysaccharides were stained as a dark mauve band on a white or pale pink background. Cellulose acetate strips keep indefinitely; paper strips deteriorate within a few hours.

2. Alcian blue.- Acidic polysaccharides were also stained as

dark blue bands against a pale blue background by dipping the paper or cellulose acetate strips in a saturated ethanolic solution of alcian blue, followed by washing in ethanol.

Zone electrophoresis of monosaccharides on Whatman No. 1 paper was carried out in 0.05M-borate buffer at pH 9.2. Papers were sprayed with aniline oxalate or p-anisidine hydrochloride containing 5% (v/v) glacial acetic acid. M_G values refer to the true migration of the sugar relative to the true migration of glucose (i.e. the observed migration distances are corrected for electroendosmotic flow movement by reference to 2,3,4,6-tetra-O-methyl-D-glucose, which does not form a borate complex).

2.7) Analytical Methods.

Nitrogen, ash and moisture determinations.- Nitrogen was determined by a semi-micro Kjeldahl method, moisture by heating to constant weight at 105°C, and ash by heating (muffle furnace) to constant weight at 550°C.

Methoxyl determinations.- A vapour-phase infrared method was used (Anderson & Duncan, 1961; Anderson, Garbutt & Zaidi, 1963).

Equivalent weight determinations on electrodialysed gum samples were carried out using standard (ca. 0.01N) sodium hydroxide solution, either potentiometrically, or titrimetrically to a phenolphthalein end-point.

Uronic acid determinations.- Uronic acid content was determined either by a vapour-phase method after decarboxylation

with hydriodic acid (Anderson et al., 1963) or by the carbazole colorimetric method (Dische, 1947, 1950). Unless otherwise stated, all values for uronic acid content are expressed as the anhydride and are corrected for moisture and protein contents.

Rhamnose determinations.- After periodate oxidation, rhamnose contents were determined as acetaldehyde by a vapour-phase infrared method (Anderson & Stoddart, 1965). See Appendix I.

Quantitative estimation of sugars.-

(a) By paper chromatography.- Sugars were separated by chromatography on thick paper sheets (Whatman No. 3MM), which had been previously soaked in 1% (v/v) acetic acid, washed with water until the washings were neutral, and dried (Knox & Hall, 1965). After elution from the paper, sugars were estimated colorimetrically by the phenol-sulphuric acid method (Dubois et al., 1956). The optical density was read on a Unicam SP 1300 spectrophotometer using filter 2. Calibration curves were constructed for known weights of sugar.

(b) By gas-liquid partition chromatography.- Galactose to arabinose ratios were also estimated from peak areas obtained on g.l.c. of their O-trimethylsilyl derivatives. The sugar mixture (10-20 mg.) was dissolved in pyridine (2 ml., anhydrous, stored over potassium hydroxide pellets); hexamethyldisilazane (0.4 ml.) and trimethylchlorosilane (0.2 ml.) were added; the mixture was shaken for 30 secs. and allowed to stand at room temperature for 1 hour. Precipitation of ammonium chloride occurred during the reaction period; this was removed

by centrifuging and a portion (2-5 µl.) of the supernatant was injected into the gas chromatograph. The retention times on column (iii) for the O-trimethylsilyl derivatives of galactose and arabinose were (T', 0.79, 0.90, 1.15) and (T', 0.23, 0.27, 0.31) respectively. A small correction factor (Ludlow et al., 1966) was determined for galactose and arabinose by comparison of the peaks produced by the O-trimethylsilyl derivatives from a mixture of equal weights of galactose and arabinose.

Unless otherwise stated, all percentages are for anhydro-sugars and are corrected for moisture and protein contents.

2.8) Preparation of derivatives.-

Preparation of phenylosazones.- The sugar (ca. 10 mg.) was heated on a boiling-water bath with phenylhydrazine (0.01 ml., distilled before use), glacial acetic acid (0.01 ml.), water (0.25 ml.), and a drop of saturated sodium bisulphite solution. On cooling and adding water (ca. 1.5 ml.), the phenylosazone precipitated; it was recrystallised from the stated solvents.

Preparation of aniline derivatives.- The O-methyl sugar (10-50 mg.) was refluxed with equimolecular amounts of freshly distilled aniline in dry ethanol (5 ml.) for 1 hour in darkness. After removal of the solvent, syrups usually crystallised; recrystallisations were carried out using the stated solvents.

Preparation of aldonolactones.- The O-methyl sugar (10-50 mg.) was oxidised with an excess of bromine water for 1 day. Excess of bromine was removed by aeration; the solution

was neutralised with silver carbonate, treated with hydrogen sulphide, filtered, and evaporated to dryness. The organic material was extracted with hot acetone:water (1:1, v/v).

Preparation of aldonamides.- The aldonolactone (10-50 mg.) was treated with dry methanolic ammonia (5 ml.) at 0°C for 1 day. After evaporation of the solvent, the aldonamide was crystallised from the stated solvents.

Melting points were taken on a Kofler hot-stage microscope; thermometers were corrected. Literature values for melting points of oligosaccharides and their derivatives were obtained from Bailey (1965); those for melting points of O-methyl sugars and their derivatives were obtained from Whistler (1965).

CHAPTER III

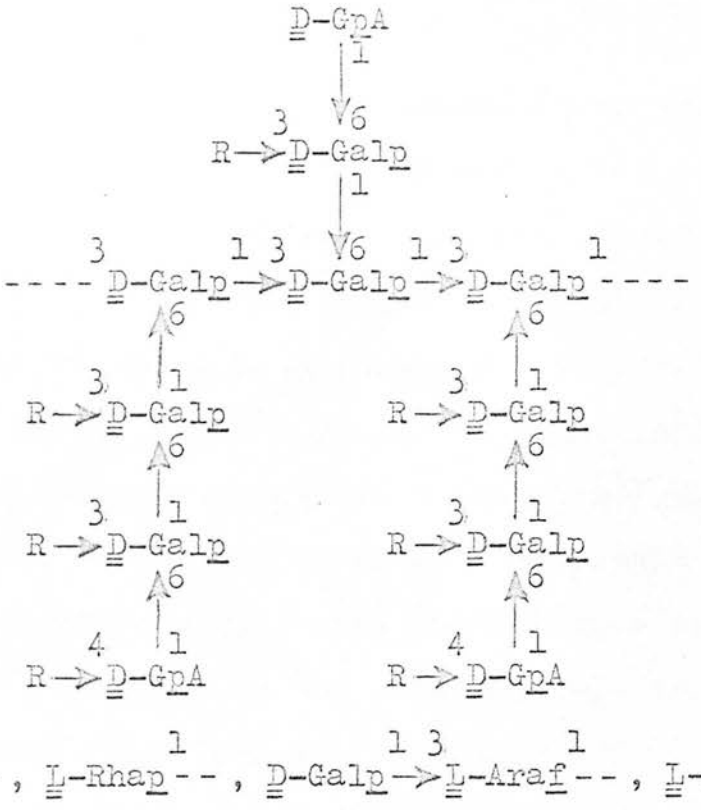
SOME STRUCTURAL FEATURES OF ACACIA SENEGAL GUM (GUM ARABIC)

3.1) Introduction.

Earlier investigations on the primary chemical structural features of Acacia senegal gum (gum arabic) have been the subject of several reviews (Hirst, 1942; Mantel, 1947; Jones & Smith, 1949; Hirst, 1951a, b; Whistler & Smart, 1953; Hirst, 1958; Hirst & Jones, 1958; Hirst, 1959; Glicksman & Schachat, 1959; Smith & Montgomery, 1959). In this early work, the gum was recognised as the salt of an acidic polysaccharide (Neubauer, 1854) containing L-arabinose (Scheibler, 1873; O'Sullivan, 1884), D-galactose (Kiliani, 1880; Claesson, 1881; O'Sullivan, 1884; Butler & Cretcher, 1929) and L-rhamnose (Norman, 1929; Smith, 1940). Prolonged acid hydrolysis of the gum has led to the isolation (Butler & Cretcher, 1929; Heidelberger & Kendall, 1929; Heidelberger, Avery & Goebel, 1929) and characterisation (Challinor, Haworth & Hirst, 1931; Hotchkiss & Goebel, 1936a,b) of the aldobiouronic acid, 6-O-(β-D-glucopyranosyluronic acid)-D-galactose. Experiments involving partial acid hydrolysis have shown that 3-O-α-D-galactopyranosyl-L-arabinose (Smith, 1939a; Jones, 1953; Charlson, Gorin & Perlin, 1957), 3-O-β-D-galactopyranosyl-D-galactose (Jackson & Smith, 1940b; Charlson et al., 1957), and

3-O-β-L-arabinopyranosyl-L-arabinose (Andrews & Jones, 1955) are constituent units of the gum.

Several important structural features have been deduced from a study of the methylated whole gum (Smith, 1940) and of the methylated degraded gum, obtained after autohydrolysis and methylation (Smith, 1939b; Jackson & Smith, 1940a). During autohydrolysis arabinofuranose residues, together with their appendages, and rhamnopyranose units are removed. Hydrolysis of the methylated degraded gum gave 2,3,4,6-tetra-, 2,3,4-tri-, and 2,4-di-O-methyl-D-galactose, and 2,3,4-tri-O-methyl-D-glucuronic acid in the approximate molar proportions 1:5:3:3. The number of possible structural formulae which could be advanced for the degraded gum was reduced by the isolation of 6-O-(2,3,4-tri-O-methyl-β-D-glucopyranosyluronic acid)-2,3,4-tri-O-methyl-D-galactose from the methylated degraded gum by controlled acid hydrolysis (Jackson & Smith, 1940a). The structural interpretations from these experiments were limited to those based on branched frameworks of 1,3- and 1,6-linked D-galactose residues. Evidence for the mode of distribution of the 1,3- and 1,6-linkages between these residues in the "core" of the gum was obtained by Dillon, O'Ceallachain & O'Colla (1953, 1954), who subjected the whole gum to three successive Barry (1943) degradations; further treatments of the degraded polysaccharide with periodate and phenylhydrazine gave a product in high yield. This indicated that little degradation had taken place during the fourth Barry degradation; it was



R = L-Araf--, L-Rhap--, D-Galp → L-Araf--, L-Arap → L-Araf--

Figure 1. Structure of gum arabic (Aspinall, Hirst & Nicolson, 1959)

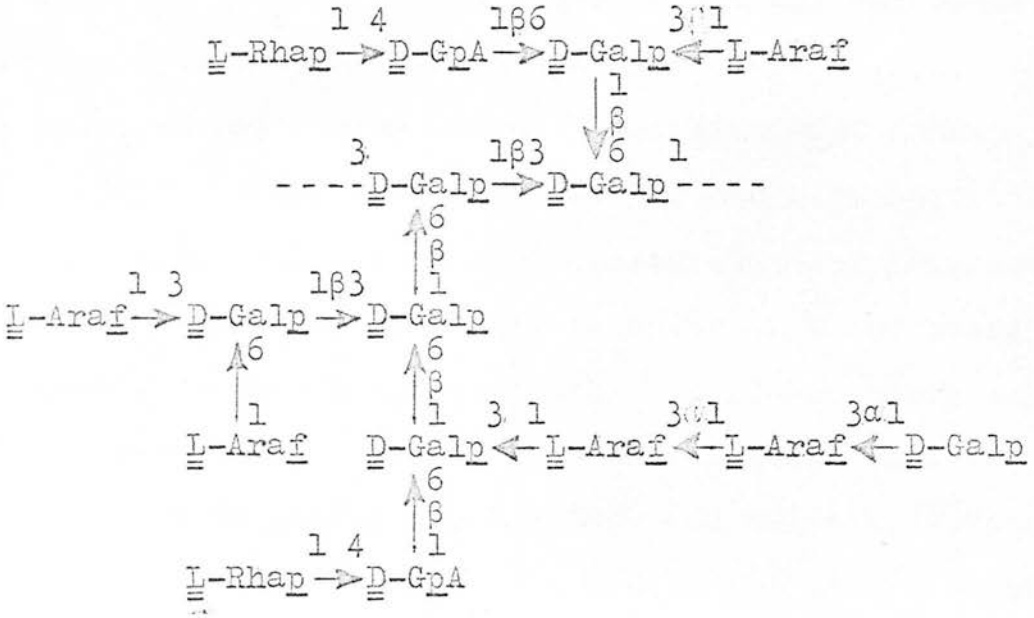


Figure 2. Main structural features of gum arabic (Hirst, 1958; Hirst & Jones, 1958; Hirst, 1959).

concluded that the gum contained a fundamental chain of D-galactose units, exclusively involving 1,3-linkages. A similar claim was made by Smith & Spriestersbach (1955), who subjected the degraded gum, obtained on autohydrolysis, to a degradation involving periodate oxidation, catalytic reduction, and a controlled acid hydrolysis; methylation of the resultant degraded galactan, followed by hydrolysis, afforded 2,3,4,6-tetra- and 2,4,6-tri-O-methyl-D-galactose; since no 2,3,4-tri-O-methyl-D-galactose was present, it was concluded that all the linkages in the galactan were of the 1,3-type. On the basis of these investigations, several polysaccharide arrays have been proposed to account for the main structural features of the gum; two of these are shown in Figures 1 and 2.

The idea that the molecular structure of A.senegal gum is based on a "main-chain" or "backbone" of D-galactose residues has been re-iterated so frequently in the literature that it may appear in a way heterodox not to accept this hypothesis. Yet, the evidence in favour of the postulate is not entirely convincing.

More recently, evidence for the attachment of some, if not all, of the L-rhamnopyranose residues to the 4-position of D-glucuronic acid units has been advanced (Aspinall, Charlson, Hirst & Young, 1963) and oligosaccharide components, obtained from carboxyl-reduced gum, have been assigned the structures, O-α-L-rhamnopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→6)-D-galactose and O-β-D-galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→3)-D-galactose (Aspinall & Young, 1965).

3.2) Results

Purification of A.senegal gum.- The gum (200 g.) was dissolved in water (4 l.), filtered, dialysed, and electrodialysed. The polysaccharide was isolated as the freeze-dried product (181 g.), $[\alpha]_D$-32° (c, 1.0)(Found: ash, 0.01%; N, 0.33%; OMe, 0.23%; equivalent weight, 1290; uronic acid, 19%; galactose, 39%; arabinose, 28%; rhamnose, 14%; sugar percentages are for the anhydro-form, and are corrected for moisture and protein contents. See Chapter II).

The gum was shown to migrate as a single band on electrophoresis on glass-fibre paper in 2N-sodium hydroxide solution, on Whatman No.1 paper in 0.1M-ammonium carbonate buffer (pH, 8.9), and on cellulose acetate film in both 0.1M-ammonium carbonate buffer (pH, 8.9) and 0.1M-acetate buffer (pH, 4.7)(See Appendix II).

Fractional precipitation of A.senegal gum with sodium sulphate.- A solution of the purified gum (25.4 g.) in water (500 ml.) was maintained at 28° C; anhydrous sodium sulphate was added in small portions with constant stirring. Precipitation commenced at concentrations approaching 40% (w/v); at 40%, a pale-brown material rose to the surface and was removed (fraction I). Two further fractions (fractions II and III), much lighter in colour, were obtained on continued, slow, stepwise addition of very small portions of sodium sulphate; eventually the supernatant contained polysaccharide material, which was not precipitated from a saturated solution of sodium

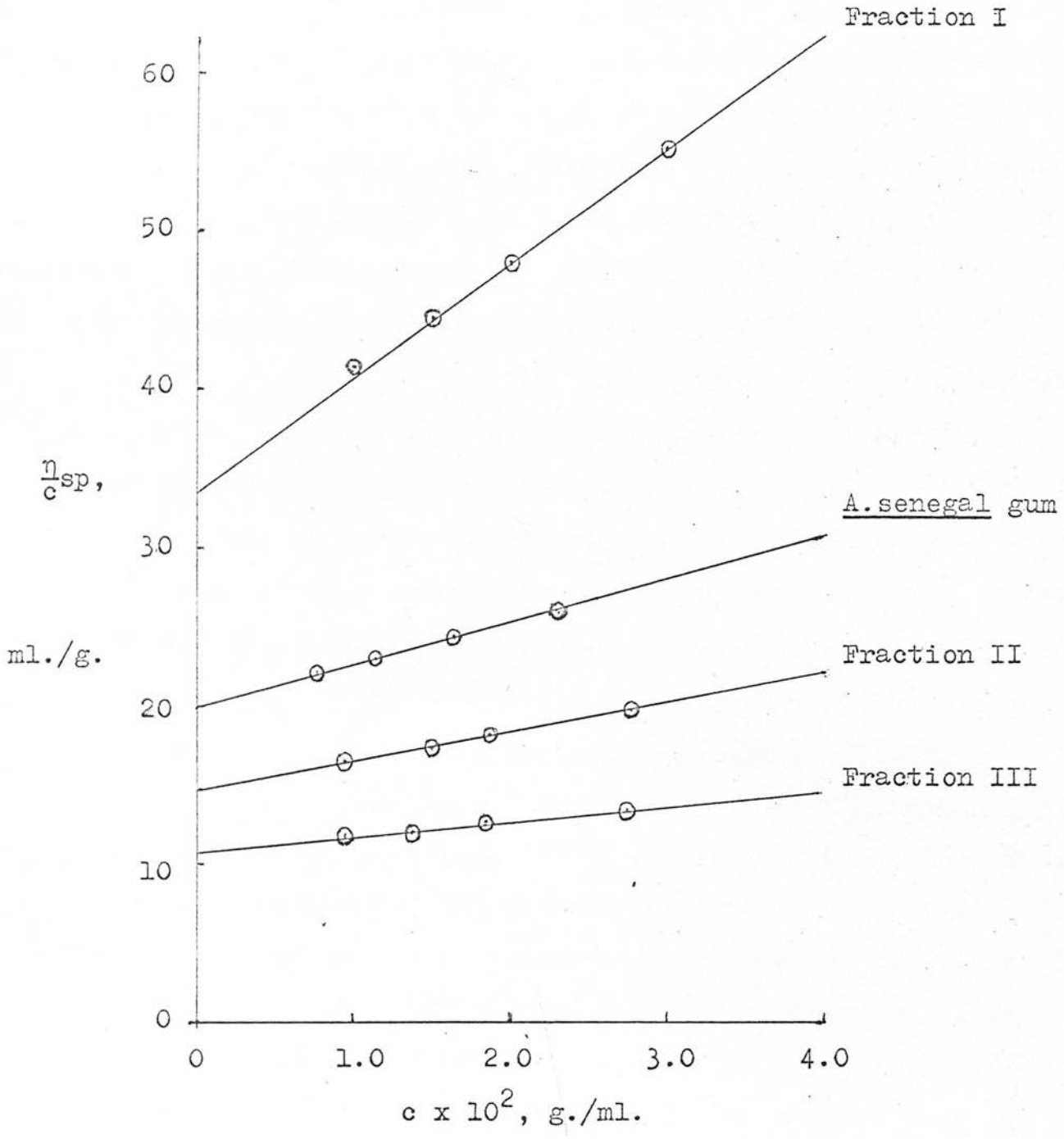


Figure 3. Plots of η_{sp}/c against c for A.senegal gum and sodium sulphate fractions I, II, and III.

sulphate, and this yielded fraction IV. The fractions were dialysed against running tap-water until free of sulphate ions; they were then electrodialysed to ensure complete removal of inorganic ions. Analytical data for the fractions are given

Table 1.

Analytical data for electrodialysed A.senegal gum and fractions obtained by precipitation with sodium sulphate.

	A.senegal gum	Fractions I	II	III	IV
Yield, %	-	23.6	29.5	33.7	1.2
Ash, %	0.01	0.01	0.02	0.01	-
Moisture, %	11.0	14.7	7.3	8.6	-
$[\alpha]_D$ (c, 1.0) [a]	-31.5°	-32.7°	-32.7°	-31.5°	-
Formic acid released on periodate oxidation for 48 hrs.(mmoles/g.) [a]	1.58	1.56	1.59	1.60	-
$[\eta]$, ml./g. [a]	20.0	33.5	14.8	10.8	-
N, %	0.33	1.01	0.12	0.02	-
Protein,% [N,% x 6.25]	2.1	6.3	0.75	0.13	-
OMe, % [b]	0.23	0.23	0.23	0.23	0.22 [c]
Uronic acid, % [b]	19	20	19	18	15.5 [c]
Rhamnose, % [b]	14	13	13	13	-
Galactose, % [b]	39	37	40	44	-
Arabinose, % [b]	28	30	28	25	-
Gal, %/Ara, % [b]	1.39	1.23	1.43	1.76	-

[a] Values are corrected for moisture contents.
[b] Values are corrected for moisture and protein contents; sugar percentages are for the anhydro-form.
[c] Values are not corrected for moisture and protein contents.

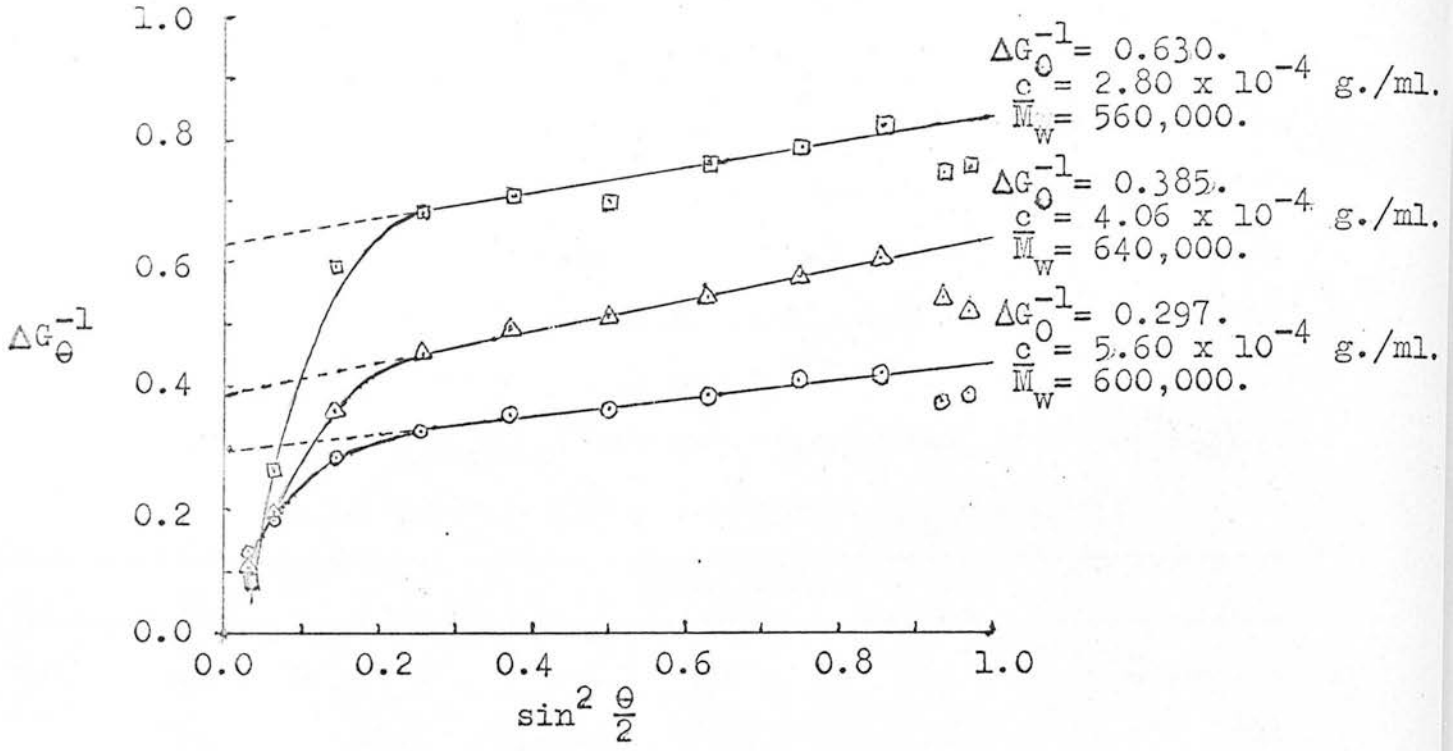


Figure 4. Plots of ΔG_θ^{-1} against $\sin^2 \frac{\theta}{2}$ for A.senegal gum.

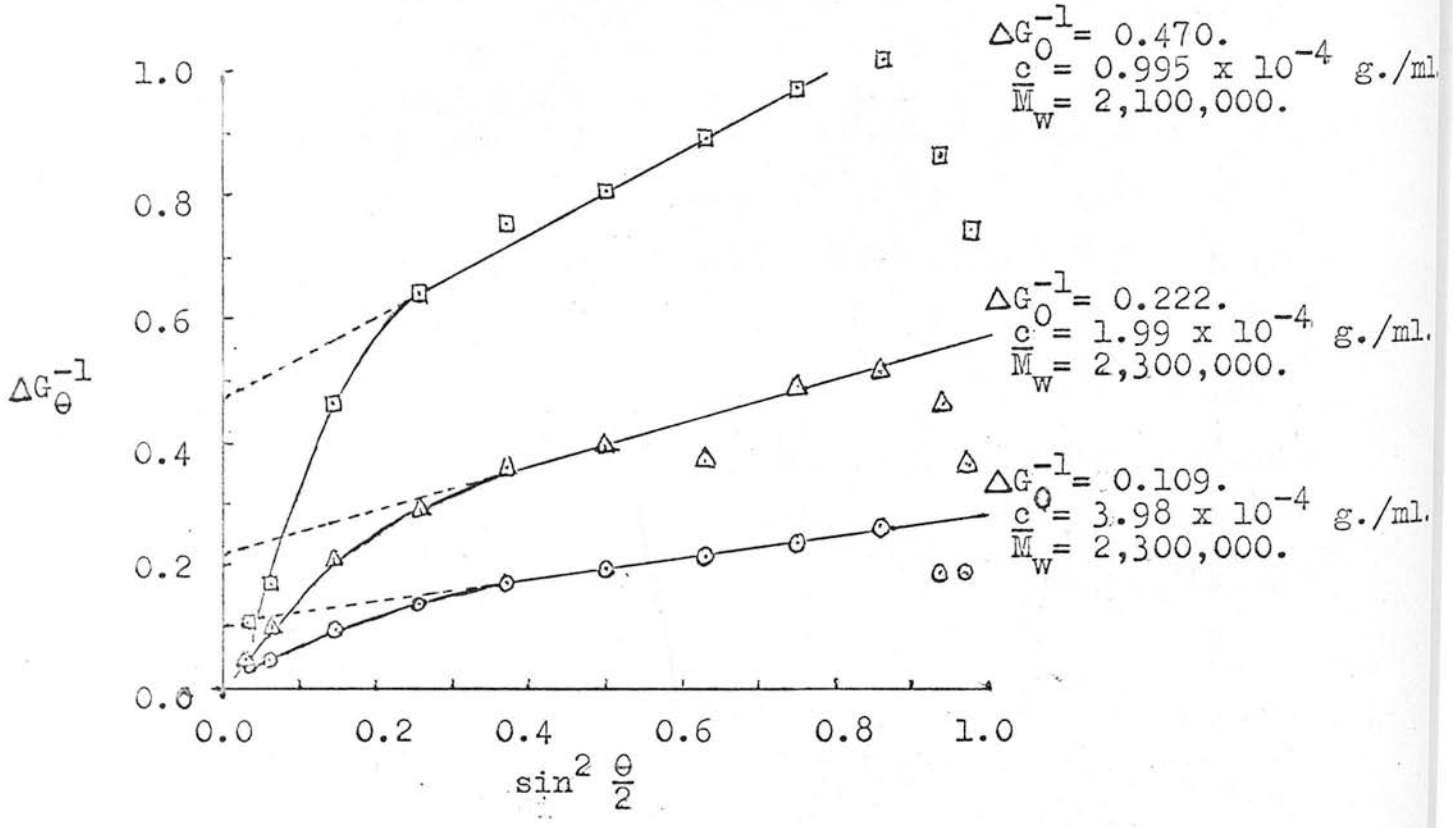


Figure 5. Plots of ΔG_θ^{-1} against $\sin^2 \frac{\theta}{2}$ for sodium sulphate fraction I.

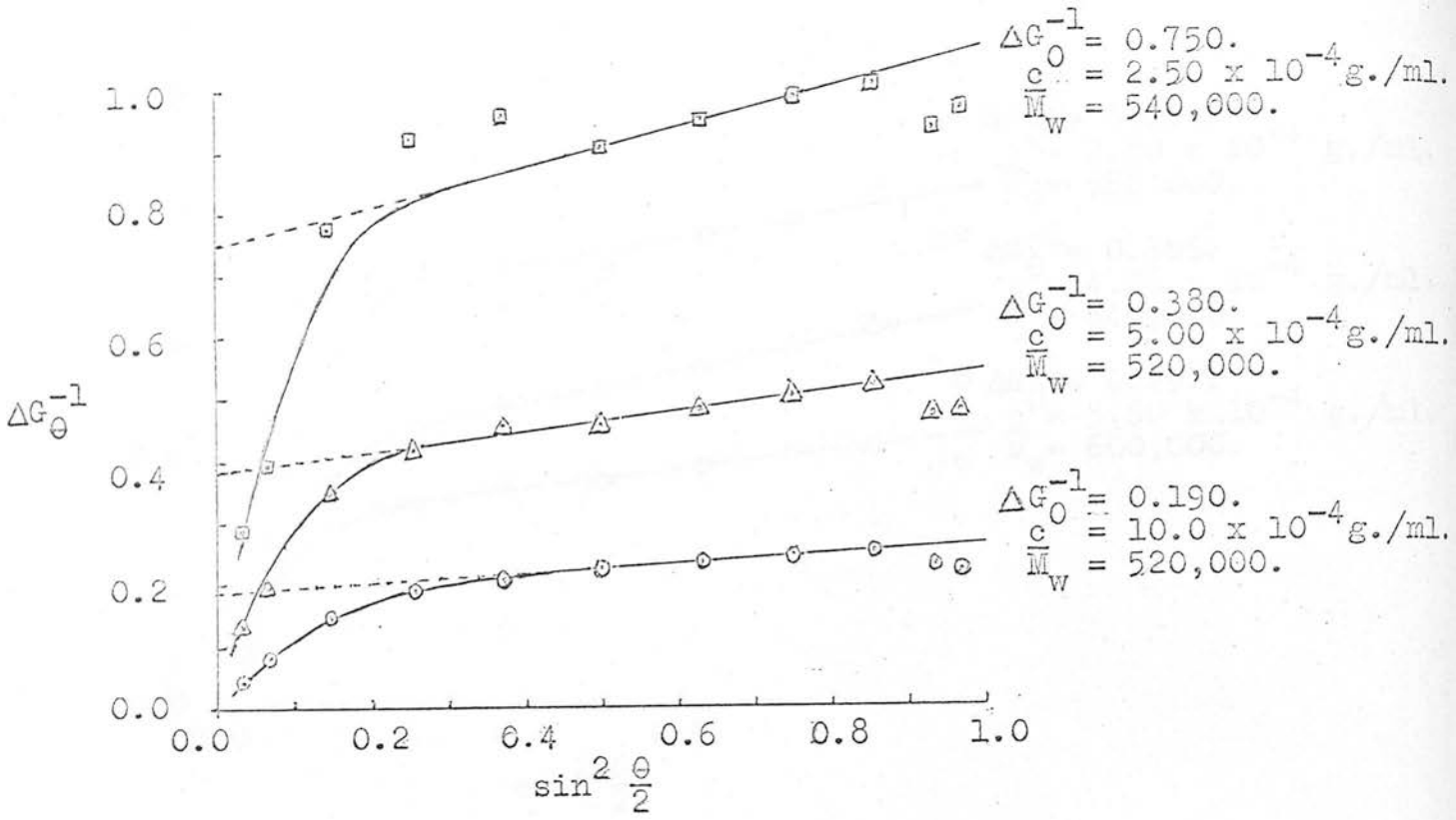


Figure 6. Plots of ΔG_θ^{-1} against $\sin^2 \frac{\theta}{2}$ for sodium sulphate fraction II.

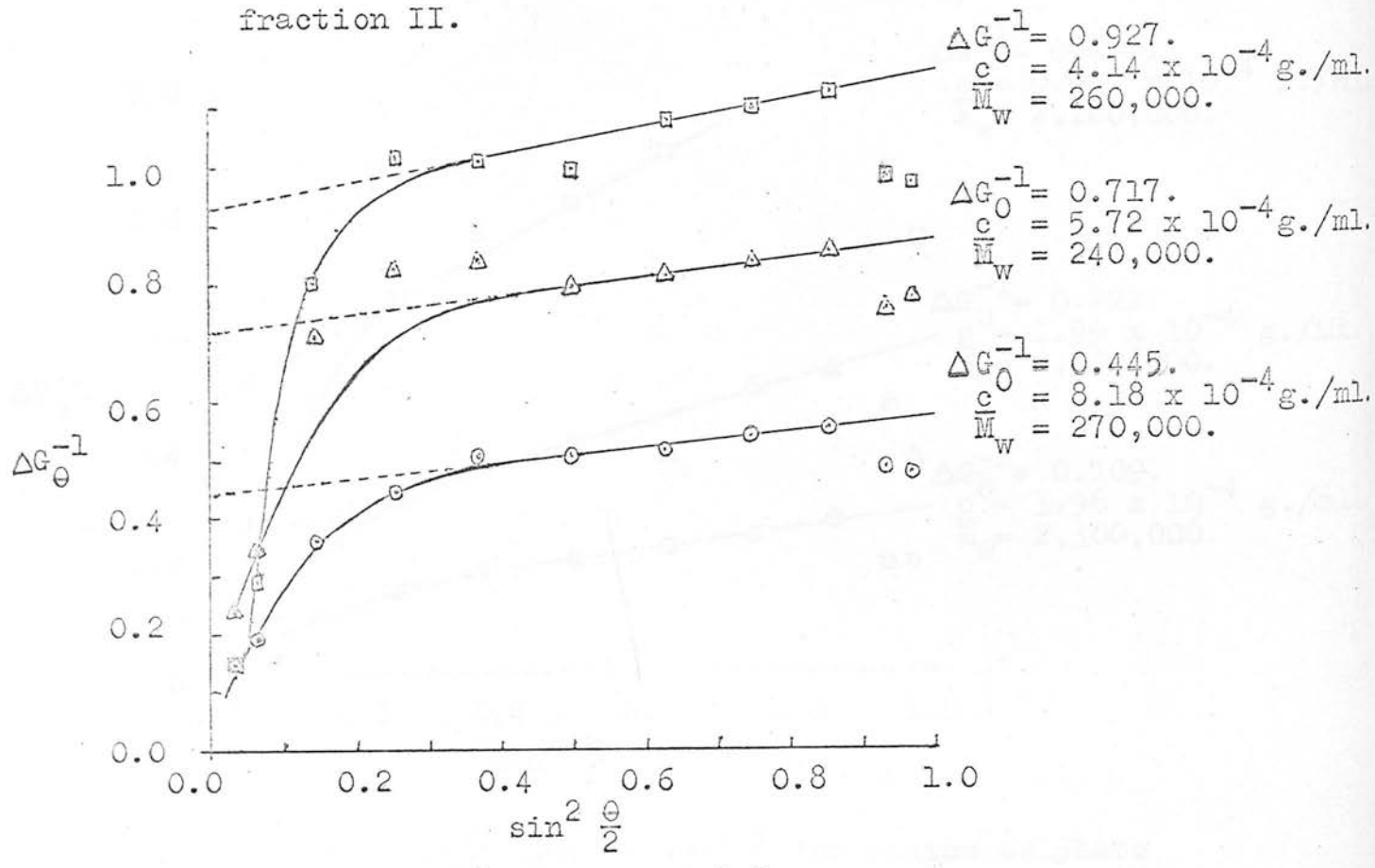


Figure 7. Plots of ΔG_θ^{-1} against $\sin^2 \frac{\theta}{2}$ for sodium sulphate fraction III.

in Table 1. All four fractions were shown to migrate as a single band on electrophoresis on cellulose acetate in 0.1M-ammonium carbonate buffer; the fractions had the same mobility as a sample of whole gum run under comparable conditions.

Viscosity and light-scattering measurements on A.senegal gum and sodium sulphate fractions.- Plots of η_{sp}/c against c for A.senegal gum, and for fractions I, II, and III, are shown in Figure 3; values for $[\eta]$, obtained from Figure 3, are recorded in Tables 1 and 2. The general equation of the straight lines shown in Figure 3 is

$$\frac{\eta}{c}sp = B'c + [\eta]$$

where B' is a constant. Values for B', calculated from the gradients, are shown in Table 2.

Plots of ΔG_{θ}^{-1} against $\sin^2 \frac{\theta}{2}$ for A.senegal gum, and for fractions I, II, and III (at three different concentrations), are shown in Figures 4,5,6, and 7, respectively. The values obtained for the $\bar{M}_w$'s of A.senegal gum, and of fractions I, II, and III, are 600,000, 2,200,000, 530,000, and 260,000, respectively (See Table 2). The $\bar{M}_w$ and $[\eta]$ values reported (Rahman, 1966; Anderson et al., 1966) for another sample of A.senegal gum, and for the sodium sulphate fractions I(a), II(a), and III(a) obtained from it, are also recorded in Table 2.

The modified (cf. Kuhn, 1934; Mark, 1938; Houwink, 1940) Staudinger (1932) equation,

$$[\eta] = K' \bar{M}_w^{a}$$

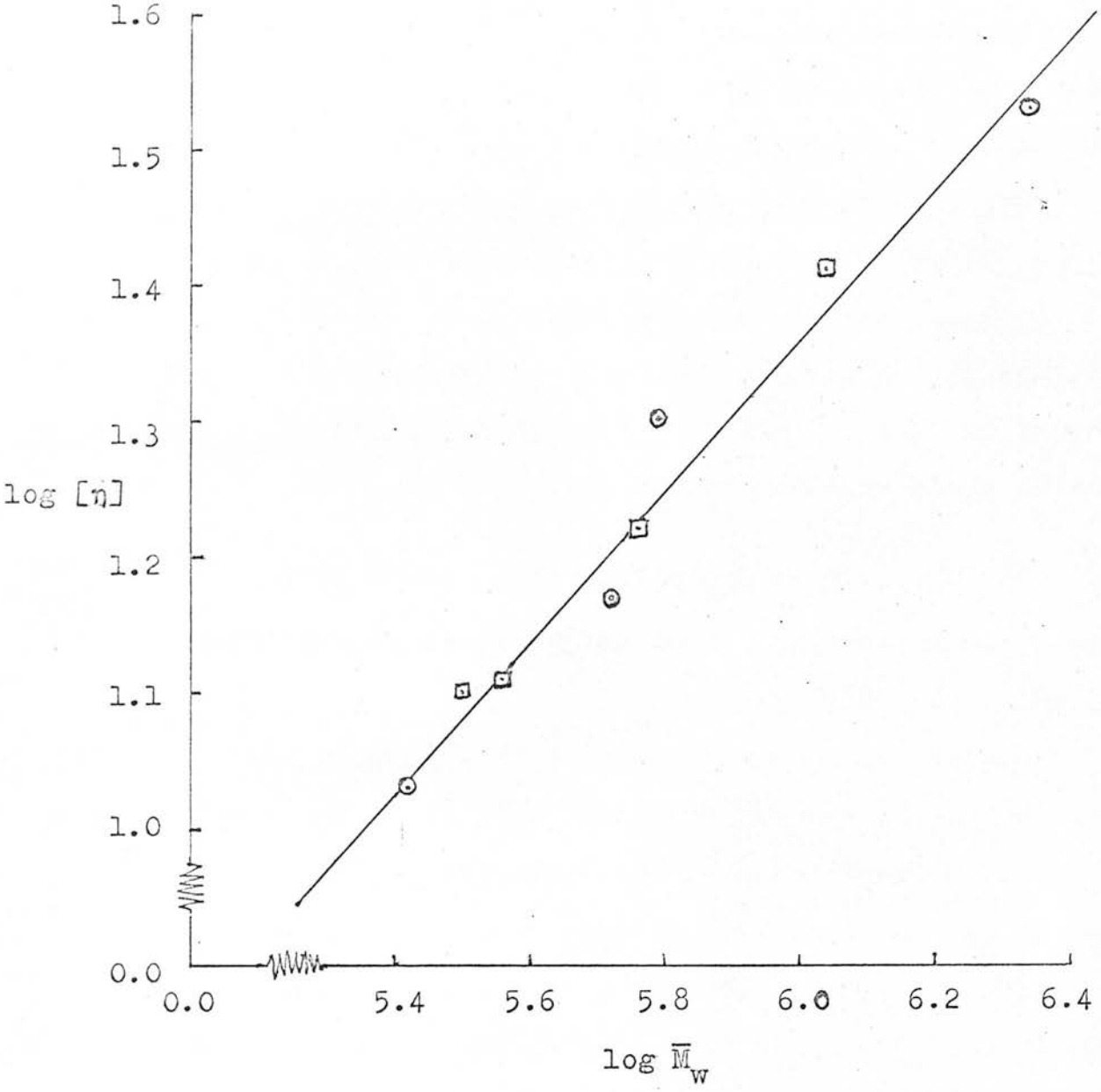


Figure 8. Plot of log [η] against log $\bar{M}_w$ for A.senegal gum and sodium sulphate fractions.

⊙ = points for A.senegal gum and fractions I,II, and III.

⊡ = points for A.senegal gum (a) and fractions I(a), II(a), and III(a) (Rahman, 1966).

relates $[\eta]$ to $\bar{M}_w$, where α and K' are constants for a particular polymer. Taking logs of both sides of this empirical equation gives

Table 2

Values for the limiting viscosity numbers and weight-average molecular-weights of two samples of A.senegal gum and their sodium sulphate fractions

	B'	$[\eta]$	$\bar{M}_w \times 10^{-6}$	$\log [\eta]$	$\log \bar{M}_w$
Fraction III	93	10.8	0.26	1.03	5.42
Fraction III (a)	-	12.5	0.32	1.10	5.50
Fraction II (a)	-	13.0	0.36	1.11	5.56
Fraction II	180	14.8	0.53	1.17	5.72
A.senegal gum (a)	-	16.4	0.58	1.22	5.76
A.senegal gum	280	20.0	0.60	1.30	5.79
Fraction I (a)	-	25.4	1.2	1.41	6.08
Fraction I	720	33.5	2.2	1.53	6.34

(a) Values obtained from Rahman (1966).

$$\log [\eta] = \alpha \log \bar{M}_w + \log K'.$$

Figure 8 shows a plot of $\log [\eta]$ against $\log \bar{M}_w$ from the data listed in Table 2. The best straight line, obtained by a least squares treatment, gives $\alpha = 0.54$ and $K' = 1.3 \times 10^{-2}$.

Methylation of A.senegal gum.- The purified gum (400 mg.) was methylated successively with dimethyl sulphate and sodium

hydroxide, and with methyl iodide and silver oxide, to give a product (340 mg.), [α] -46° (c, 1.0 in $CHCl_3$)(Found: OMe, 41.9%, not raised on further attempted methylation). Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 3; retention times on columns (i) and (ii) were comparable with those for methyl glycosides from authentic O-methyl sugars. Figure 9 records a typical gas chromatogram obtained from column (ii). A portion of the mixture of methyl glycosides was hydrolysed with N-sulphuric acid for 3 hours on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated. Examination of the syrup by paper chromatography in solvents (c) and (f) showed the presence of 2-O-methylgalactose in addition to those O-methyl sugars already characterised by g.l.c. of their methyl glycosides (Table 3).

Autohydrolysis of A.senegal gum.- A sample (4 g.) of electrodialysed gum was dissolved in water to give a 2% (w/v) solution (pH, 2.8). Autohydrolysis on a boiling-water bath was followed polarimetrically (Smith, 1939a). After 50 hours, the solution was cooled, filtered (to remove denatured protein), and dialysed against water (3 x 2 l.). Dialysis was completed against running tap-water, and freeze-drying gave the autohydrolysed gum (2 g.), $[\alpha]_D$-11° (c, 1.0)(Found: OMe, 0.25%, not reduced on attempted saponification with N-sodium hydroxide solution; uronic acid, 21%; galactose, 77%; arabinose, 2%).

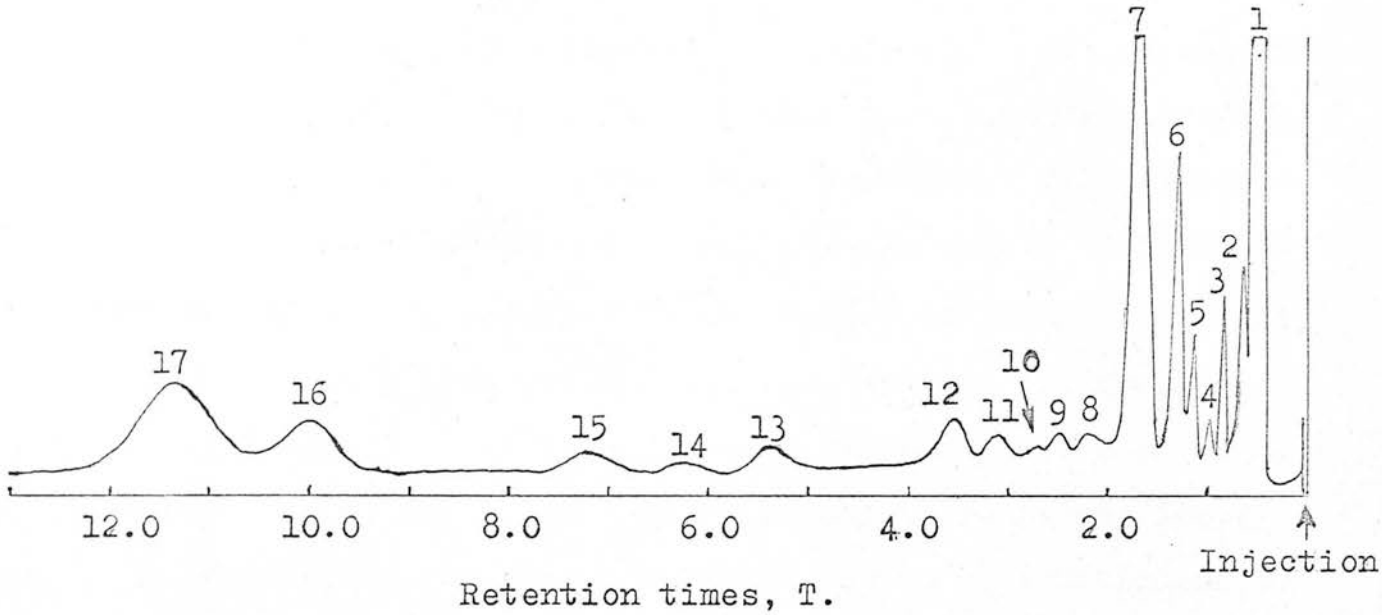


Figure 9. Gas chromatogram [column (ii)] of the methanolysis products from methylated Acacia senegal gum.

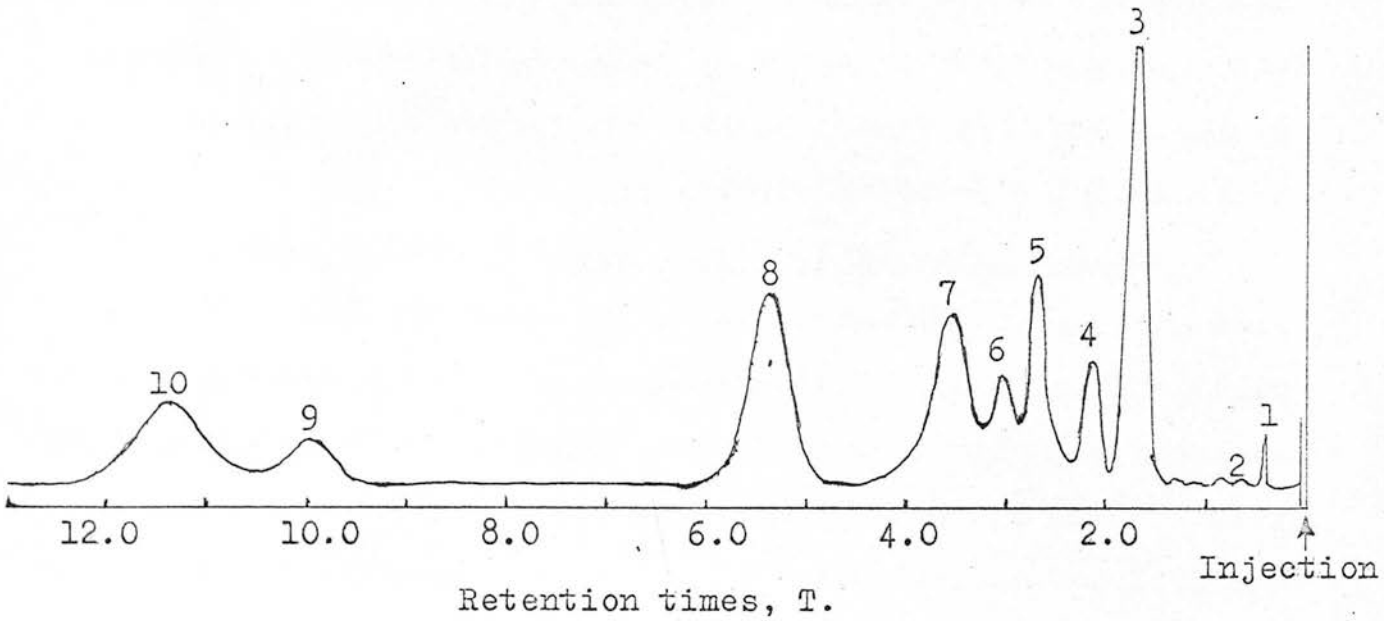


Figure 12. Gas chromatogram [column (ii)] of the methanolysis products from methylated autohydrolysed gum.

Table 3.

Examination of methanolysis and hydrolysis products from methylated A.senegal gum

O-Methyl Sugars	Relative retention times, T, of methyl glycosides on columns:- (i)	(ii)	Peak numbers [a]	R_G in solvent (c)
2,3,4-Me_3-rhamnose	0.49	(0.51)	1	1.01
2,3,5-Me_3-arabinose	0.58	(0.51)	1	0.98
	0.72	0.64	2	
2,3,4-Me_3-arabinose	0.96	0.84	3	0.78
2,5-Me_2-arabinose	1.78	1.27	6	0.80
	3.29	(2.21) [b]	8	
2,3,4,6-Me_4-galactose	1.68	1.67	7	0.87
2,4,6-Me_3-galactose	3,74	3.05	11	0.73
	4.18	3.48	12	
2,3,4-Me_3-galactose	6.42	5.27	13	0.73
2,4-Me_2-galactose	14.6	9.8	16	0.53
	16.5	11.0	17	
2,3,4-Me_3-glucuronic acid [c]	2.23	(2.21)	8	-
	2.96	2.73	10	
2,3-Me_2-glucuronic acid [c]	7.8	6.2	14	-
	9.3	7.1	15	
2-Me-galactose	-	-	-	0.32
Unknown sugars	1.17	0.98	4	-
	1.44	1.15	5	-
	-	2.48	9	-

[a] Peak numbers refer to those in Figure 9.

[b] Figures in parentheses indicate T values of components not completely resolved.

[c] As methyl ester methyl glycoside.

Hydrolysis of the autohydrolysed gum on a boiling-water bath with N-sulphuric acid for 7 hours indicated the presence of two aldobiouronic acids, which had R_{gal} values of 0.22 (major component) and 0.59 (minor component) in solvent (b); they were chromatographically identical to 6-O-(β-D-glucopyranosyluronic acid)-D-galactose and 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose, respectively. Paper chromatographic examination of the diffusate from the autohydrolysed gum showed it to contain galactose, arabinose, rhamnose, three (major) neutral disaccharides, traces of two aldobiouronic acids, and oligosaccharide material. Further hydrolysis of a portion of the diffusate yielded more of the same aldobiouronic acids found in the autohydrolysed gum.

Molecular-sieve chromatography of A.senegal gum, sodium sulphate fractions, and autohydrolysed gum.- Figure 10 shows a calibration plot of elution volume, V_e, against log $\bar{M}_n$ obtained with dextran fractions of known $\bar{M}_n$. Elution volumes for dextran fractions in separate experiments on the same column were reproducible within reasonably narrow limits (i.e. 5-15 ml.); differences of a few ml. in the elution volumes were possibly a result of small fluctuations in temperature. For "Bio-Gel P300,"

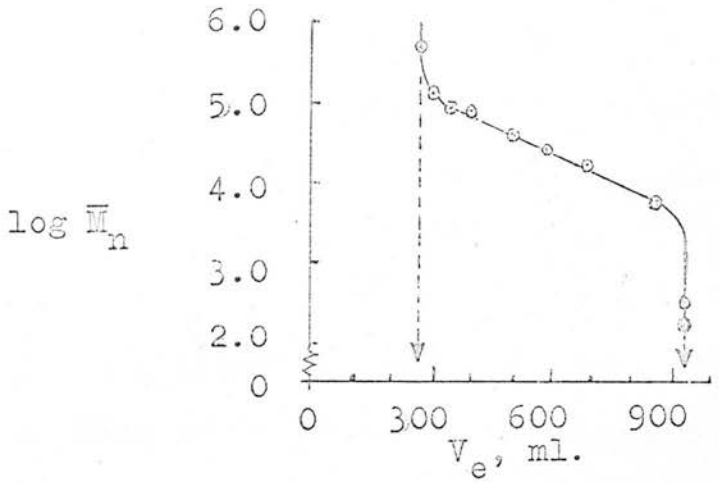


Figure 10. Plot of elution volume, V_e, against log $\bar{M}_n$ for dextran fractions of known $\bar{M}_n$ values. "Bio-Gel P300" column (50 x 4.8 cm.): elution with M-sodium chloride. The arrows correspond with those on Figure 11.

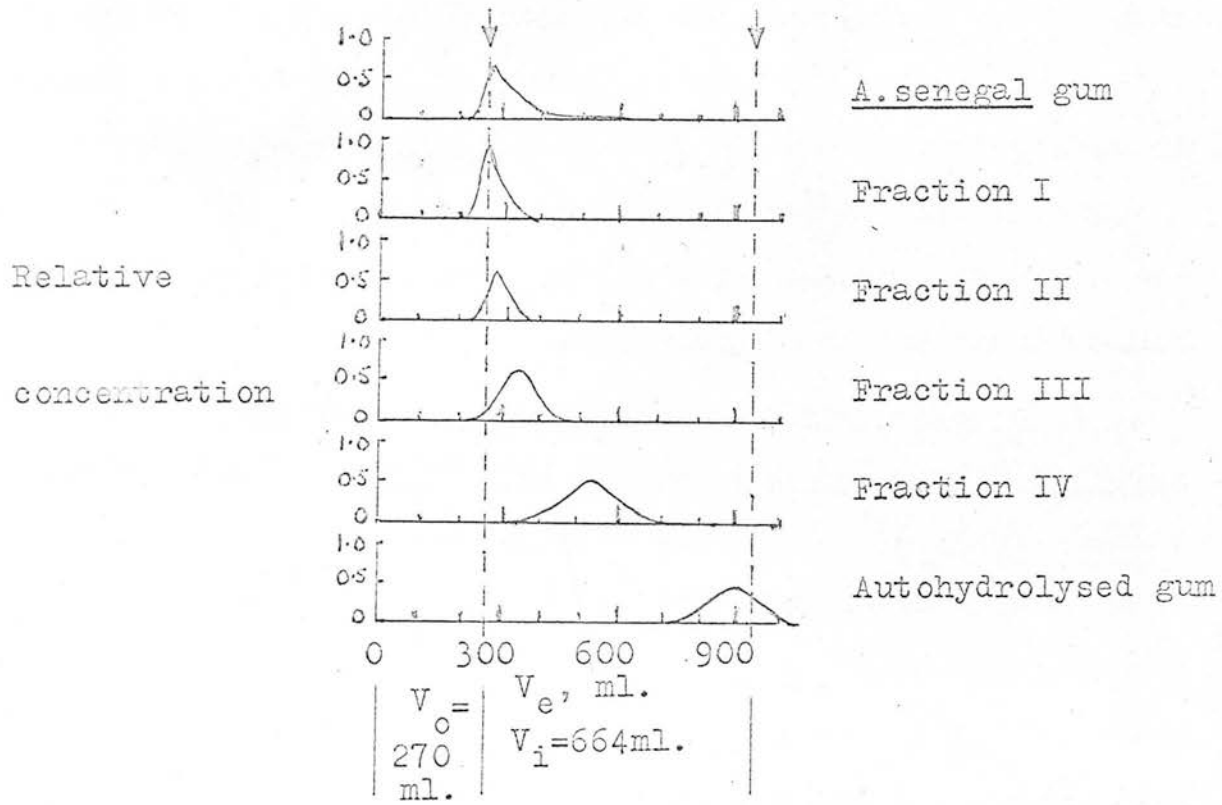


Figure 11. Elution patterns for A. senegal gum, sodium sulphate fractions, and autohydrolysed gum. "Bio-Gel P300" column (50 x 4.8): elution with M-sodium chloride.

a plot of V_e against log $\bar{M}_n$ (Andrews, 1962, 1964, 1965) is approximately linear for values of $\bar{M}_n$ from 5,000-125,000; although the useful working range may extend slightly beyond these values, the exclusion limit of "Bio-Gel P300" for the polysaccharides investigated is apparently less than 300,000. (An exclusion limit of 300,000 molecular-weight is claimed by the manufacturers for proteins). Sucrose and glucose have the same elution volume, which is defined as being equal to $V_o + V_i$,

Table 4.

Estimation of $\bar{M}_n$ by molecular-sieve chromatography.

	V_e	K_d	$\bar{M}_n$
A.senegal gum	(276)	-	-
Fraction I	270	0.00	-
Fraction II	297	0.04	140,000 ± 20,000
Fraction III	351	0.12	99,000 ± 10,000 (105,000)[a]
Fraction IV	532	0.40	35,000 ± 3,000 (37,000)[a]
Autohydrolysed gum	884	0.92	4,800 ± 500 (4,400)[b]

[a] I wish to thank Dr. S. Rahman for carrying out these determinations by osmometry.

[b] Periodate end-group analysis as formaldehyde.

where V_o is the void volume, and V_i is the internal volume (Andrews, 1964; Anderson & Stoddart, 1966b). The elution volume of "blue dextran" was taken as the void volume, and values for

the distribution coefficient, K_d, were calculated from the relationship (Gelotte, 1960), $K_d = (V_e - V_o)/V_i$. Figure 11 shows the elution patterns obtained for A.senegal gum, for Fractions I-IV precipitated by sodium sulphate, and for autohydrolysed gum. Table 4 gives the values found for K_d and $\bar{M}_n$; estimation of $\bar{M}_n$ for the whole gum was rendered difficult by the asymmetric nature of its elution curve.

Methylation of autohydrolysed gum.- The autohydrolysed gum (200 mg.) was methylated successively with dimethyl sulphate

Table 5.

Examination of methanolysis and hydrolysis products from methylated autohydrolysed gum.

O-Methyl sugars	Relative retention times, T, of methyl glycosides on columns:- (i)	(ii)	Peak[a] numbers	R_G in solvent (c)	Approx. relative molar props.
2,3,5-Me_3-arabinose	0.58	0.46	1	-	Trace
	0.72	0.64	2		
2,3,4,6-Me_4-galactose	1.67	1.64	3	0.88	++
2,4,6-Me_3-galactose	3.73	2.99	6	0.72	++
	4.18	3.46	7		
2,3,4-Me_3-galactose	6.42	5.22	8	0.72	+++
2,4-Me_2-galactose	14.7	9.9	9	0.52	+++
	15.9	11.2	10		
2,3,4-Me_3-glucuronic acid[b]	2.31	2.09	4	-	+++
	2.94	2.68	5		
2-Me—galactose	-	-	-	0.34	+

[a] Peak numbers refer to those in Figure 12.

[b] As methyl ester methyl glycoside.

and sodium hydroxide, and with methyl iodide and silver oxide, to give a product (82 mg.), $[\alpha]_D$ -56° (c, 1.0 in $CHCl_3$)(Found: OMe, 42.1%, not raised on further attempted methylation). The methylated polysaccharide (50 mg.) was refluxed for 8 hours with methanolic 5% hydrogen chloride (10 ml.). After neutralisation with silver carbonate, and filtration, the methanolic solution was concentrated to a syrup on a rotary evaporator. G.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 5; Figure 12 records a typical gas chromatogram obtained from column (ii). Examination of a hydrolysate of the methyl glycosides by paper chromatography in solvents (c) and (f) showed the presence of 2-O-methylgalactose in addition to those O-methyl sugars already characterised as their methyl glycosides by g.l.c. (Table 5).

Borohydride reduction of autohydrolysed gum.- Autohydrolysed gum (500 mg.) was dissolved in water (100 ml.) and sodium borohydride (400 mg.) was added. The solution was kept for 24 hours at room temperature before further sodium borohydride (100 mg.) was added. After the solution had been stirred for 6 hours, it was dialysed against running tap-water for 2 days. The freeze-dried product was hydrolysed to yield the same aldobiouronic acids and neutral disaccharides as found in the autohydrolysed gum. In addition, paper chromatography in

solvent (h) and t.l.c. on silica gel with solvent (j) indicated the presence of galactitol. No arabinitol was detected.

Periodate oxidation of autohydrolysed and reduced autohydrolysed gum.- Autohydrolysed gum did not give detectable amounts of formaldehyde on periodate oxidation. Reduced autohydrolysed gum (34.32 mg., dry wt.), dissolved in a 0.1% (w/v) solution (10 ml.) of p-hydroxybenzaldehyde, which prevents recombination of formaldehyde with oxypolysaccharide (O'Dea, 1953), was oxidised with 0.3M-sodium metaperiodate solution (1 ml.). At suitable time intervals, samples (1 ml.) were transferred to centrifuge tubes and treated with 0.5M-sodium sulphite solution (1 ml.) to destroy excess of periodate, and with ethanol (4 ml.) to precipitate the oxypolysaccharide; the tubes were stored for 2 days at 2°C and then centrifuged. Portions (1 ml.) were treated with 9 ml. chromotropic acid reagent [2 g. "sodium salt for formaldehyde determinations" (B.D.H. Ltd.) dissolved in a solution of AnalaR sulphuric acid (556 ml.) and water (320 ml.)](Hay, 1964) on a boiling-water bath for 30 mins. After cooling, 4.6% (w/v) thiourea solution (2 ml.) was added, and the optical density was measured against a blank on a Unicam SP 1300 (Filter 4). Calibration curves for formaldehyde were constructed by periodate oxidation of AnalaR glucose solutions, 0.5M with respect to sodium bicarbonate. The production of formaldehyde with time from reduced autohydrolysed gum was as follows: 0.25 hr., 160 μg.; 0.5 hr., 200 μg.; 1 hr., 215 μg.; 2 hr., 220 μg.; 6 hr., 220 μg.; 24 hr., 235 μg. Assuming

production of one formaldehyde molecule per average polymer unit, a value of 4,400 for the $\bar{M}_n$ of autohydrolysed gum was calculated. Taking into account its chemical composition, this corresponds to a number-average degree of polymerisation, $\bar{P}_n$, of about 27.

Controlled Smith-degradation of autohydrolysed gum.- (Goldstein, Hay, Lewis & Smith, 1959; Smith & Montgomery, 1959).- Periodate oxidation was carried out at 2°C. Autohydrolysed gum (1 g.) was dissolved in water (25 ml.), 50% (w/v) periodic acid (1.75 ml.) was added, and the solution made up to 50 ml. After 2 days, the reaction was stopped by addition of excess of ethylene glycol. Following dialysis against running tap-water for 2 days, the oxypolysaccharide was reduced with sodium borohydride (250 mg.) for 36 hours. Further dialysis for 2 days was followed by hydrolysis of the acetal linkages with N-sulphuric acid for 2 days at room temperature. The acidic solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated. Paper chromatographic examination of the syrupy material revealed the presence of glycerol and glycollic aldehyde. Molecular-sieve chromatography on a column (75 x 2.5 cm.) of "Bio-Gel P10" was used to separate such low molecular-weight materials from the Smith-degraded product (180 mg.). Paper chromatographic examination of a hydrolysate of a small portion (30 mg.) of this product indicated the presence of galactose, arabinose (a trace) and arabinitol [solvent (h)], but no galactitol or erythritol.

Methylation of Smith-degraded autohydrolysed gum.- The Smith-degraded autohydrolysed gum (100 mg.) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give a product (22 mg.) (Found: OMe, 36.2%). Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 6; Figure 13 records a typical gas chromatogram obtained from column (ii). Hydrolysis of the mixture of methyl glycosides, followed by paper chromatographic examination in solvents (c) and (f) showed the presence of 2-O-methylgalactose in addition to those O-methyl sugars already characterised by g.l.c. of their methyl glycosides (Table 6).

Preparation and methylation of polysaccharide A.- The purified gum (20.0 g., dry wt.) was dissolved in water (500 ml.) and 0.25M-sodium metaperiodate solution (500 ml.) was added. The oxidation (in darkness at room temperature) was followed by measuring the release of formic acid with time; the results are shown in Table 7. After 48 hours, the reaction was stopped by addition of ethylene glycol (10 ml.); the solution was dialysed against running tap-water for 2 days. Sodium borohydride (5 g.) was added and the mixture kept at room temperature for 30 hours, then dialysed for a further 2 days. The polyalcohol was hydrolysed in N-sulphuric acid at room temperature for 2 days after which the solution was neutralised (barium carbonate), filtered, deionised [Amberlite resin IR-120 (H)], reduced in volume to ca. 250 ml., and dialysed against water (1 l.). The

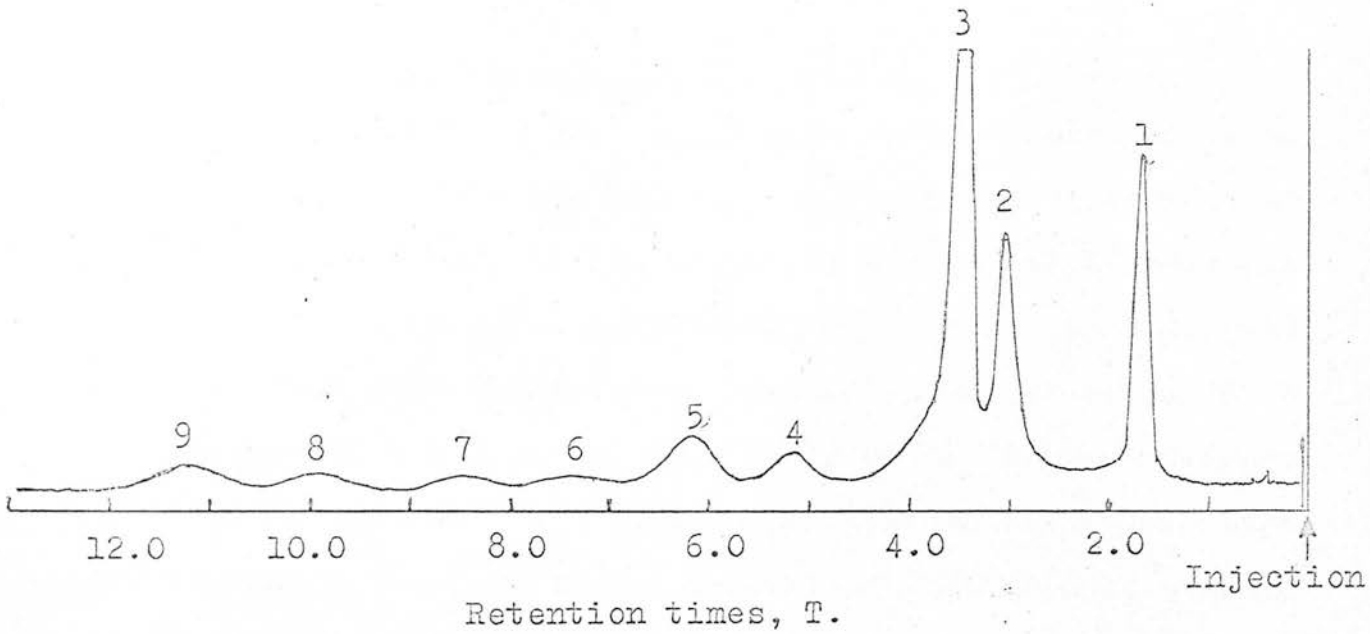


Figure 13. Gas chromatogram [column (ii)] of the methanolysis products from methylated Smith-degraded autohydrolysed gum.

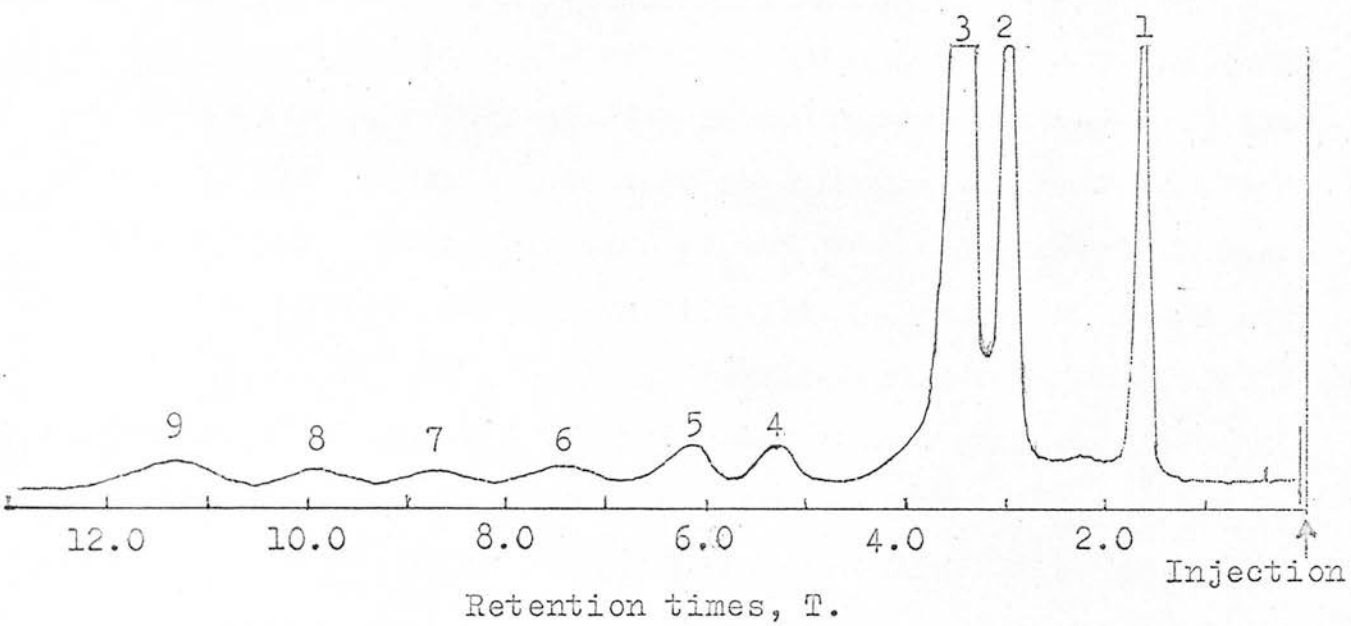


Figure 14. Gas chromatogram [column (ii)] of the methanolysis products from methylated polysaccharide E.

Table 6.

Examination of methanolysis and hydrolysis products from methylated Smith-degraded autohydrolysed gum.

O-Methyl sugars	Relative retention times, T, of methyl glycosides on columns:- (i)	(ii)	Peak[a] numbers	R_G in solvent (c)	Approx. relative molar props.
2,3,4,6-Me_4-galactose	1.66	1.65	1	0.87	+
2,4,6-Me_3-galactose	3.74	3.02	2	0.73	++++
	4.14	3.48	3		
2,3,4-Me_3-galactose	6.35	5.21	4	0.73	Trace
2,6-Me_2-galactose	9.2	6.2	5	0.54	+
		7.5	6		
		8.5	7		
2,4-Me_2-galactose	14.6	9.9	8	0.50	+/2
	16.0	11.3	9		
2-Me -galactose	-	-	-	0.33	+/2

[a] Peak numbers refer to those in Figure 13.

Table 7.

Formic acid released (mmoles/g.) on periodate oxidations.

Time (hours)	1	3	4	6	11	12	24	48	72	96
A.senegal gum	0.73	0.99	-	1.14	-	1.44	1.49	1.58	-	-
Polysaccharide A	0.80	1.21	-	1.55	1.74	-	2.12	2.74	3.17	3.26
Polysaccharide B	1.18	1.19	-	-	-	1.22	1.23	-	-	-
Polysaccharide C	1.10	-	1.18	1.21	-	-	1.23	-	-	-

syrup obtained from the dialysate was shown by paper chromatography to contain glycerol and glycollic aldehyde as the main components, together with trace amounts of arabinose, ethylene glycol, and some slower moving non-reducing glycosides. After dialysis against running tap-water for 2 days, polysaccharide A (13.6 g., dry wt., yield, 69%), $[\alpha]_D$-28° (c, 1.0) (Found: uronic acid, 4%, by decarboxylation; galactose, 69%; arabinose, 27%) was isolated as the freeze-dried product. Hydrolysis of the polysaccharide with N-sulphuric acid for 7 hours on a boiling-water bath gave a trace of the aldobiouronic acid, 6-O-(β-D-glucopyranosyluronic acid)-D-galactose [R_{gal}, 0.21 in solvent (b)] in addition to galactose and arabinose.

Polysaccharide A (300 mg.) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give a product (213 mg.), $[\alpha]_D$-41° (c, 1.0 in $CHCl_3$)(Found: OMe, 39.4%, not raised on further attempted methylation). Methanolysis of a sample of this product,

followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 8.

Table 8.

O-Methyl sugars present in methylated polysaccharides A - E.

O-Methyl sugars	A	B	C	D	E
2,3,5-Me_3-arabinose	+	+	+		
2,5-Me_2-arabinose	+	+			
2,3,4,6-Me_4-galactose	+	+	+	+	+
2,4,6-Me_3-galactose	+	+	+	+	+
2,3,4-Me_3-galactose	+	+	+	+	+
2,6-Me_2-galactose	+	+	+	+	+
2,4-Me_2-galactose	+	+	+	+	+
2-Me—galactose	+	+	+		
2,3,4-Me_3-glucuronic acid	+				

Examination of a hydrolysate of the mixture of methyl glycosides by paper chromatography in solvents (c) and (f) showed the presence of 2-O-methylgalactose, in addition to those O-methyl sugars already characterised by g.l.c. of their methyl glycosides (Table 8).

Preparation and methylation of polysaccharide B.- Borohydride reduction of periodate-oxidised polysaccharide A (12.0 g., dry wt.), followed by controlled acid hydrolysis at room temperature with N-sulphuric acid for 2 days, gave polysaccharide B

(6.4 g., dry wt., yield, 54%), $[\alpha]_D$-9° (c, 1.0)(Found: galactose, 89%; arabinose, 11%). (The N-sulphuric acid solution was not neutralised with barium carbonate in this case, nor in subsequent Smith degradations; degraded polysaccharides were isolated after dialysis of the acidic solutions against running tap-water for 2 days. This procedure was adopted, because polysaccharide material, which often precipitated from acidic solutions, redissolved during dialysis). The release of formic acid on periodate oxidation of polysaccharide A is shown in Table 7.

Polysaccharide B was methylated (procedure as for polysaccharide A) to give a product (296 mg.), $[\alpha]_D$-31° (c, 1.0 in $CHCl_3$)(Found: OMe, 39.0%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 8. Examination of a hydrolysate of the mixture of methyl glycosides by paper chromatography in solvents (c) and (f) showed the presence of 2-O-methylgalactose, in addition to those O-methyl sugars already characterised by g.l.c. of their methyl glycosides (Table 8).

Preparation and methylation of polysaccharide C.- Borohydride reduction of periodate-oxidised polysaccharide B (5.0 g., dry wt.), followed by controlled acid hydrolysis with N-sulphuric acid for 2 days at room temperature, gave polysaccharide C (3.1 g., dry wt., yield, 63%), $[\alpha]_D$ +14° (c, 1.0)(Found: galactose, 98%; arabinose, 2%). Results for release of formic

acid on periodate oxidation of polysaccharide B are shown in Table 7.

Polysaccharide C (204 mg.) was methylated (procedure as for polysaccharide A) to give a product (104 mg.), $[\alpha]_D$-12° (c, 1.0 in $CHCl_3$) (Found: OMe, 38.1%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 8. Examination of a hydrolysate of the mixture of methyl glycosides by paper chromatography in solvents (c) and (f) showed the presence of 2-O-methylgalactose, in addition to those O-methyl sugars already characterised by g.l.c. of their methyl glycosides (Table 8).

Preparation, partial acid hydrolysis, and methylation of polysaccharide D.- Borohydride reduction of periodate-oxidised polysaccharide C (2.5 g., dry wt.), followed by controlled hydrolysis with N-sulphuric acid for 2 days at room temperature, gave polysaccharide D (1.55 g., dry wt., yield, 64%), $[\alpha]_D$ +25° (c, 1.0), which contained only galactose. Results for release of formic acid on periodate oxidation of polysaccharide C are shown in Table 7.

Polysaccharide D (200 mg.) was hydrolysed with 0.5N-sulphuric acid (10 ml.) for 1 hour on a boiling-water bath; after cooling, the solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated. Paper chromatographic examination of the syrup in solvent (e) indicated the presence of galactose, two

neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose (R_{gal} 0.54, major component) and 6-O-β-D-galactopyranosyl-D-galactose (R_{gal} 0.37, minor component), and higher neutral oligosaccharides including the β1,3-linked galactotriose (R_{gal} 0.25).

Polysaccharide D (93 mg.) was methylated (procedure as for polysaccharie A) to give a product (42 mg.), $[\alpha]_D$ -1° (c, 0.84 in $CHCl_3$) (Found: OMe, 36.7%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 8.

Preparation, partial acid hydrolysis, and methylation of polysaccharide E.- Borohydride reduction of periodate-oxidised polysaccharide D (500 mg., dry wt.), followed by controlled hydrolysis with N-sulphuric acid for 2 days at room temperature, yielded a galactan, polysaccharide E (322 mg., dry wt., yield, 67%, $[\alpha]_D$ +30° (c, 1.0). The amount of formic acid released on periodate oxidation of polysaccharide D over 24 hours was 0.57 mmoles/g.

Partial acid hydrolysis of polysaccharide E (10 mg.), followed by paper chromatographic examination of the hydrolysate indicated the presence of galactose and the same neutral oligosaccharides as found in polysaccharide D.

Polysaccharide E (89 mg.) was methylated (procedure as for polysaccharide A) to give a product (38 mg.), $[\alpha]_D$ -1° (c, 0.76 in $CHCl_3$) (Found: OMe, 36.5%, not raised on further attempted

methylation). Methanolysis, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Tables 8 and 9; Figure 14 records a typical gas chromatogram obtained from column (ii). Examination of a hydrolysate of

Table 9.

Examination of methanolysis and hydrolysis products from methylated polysaccharide E.

O-Methyl sugars	Relative retention times, T, of methyl glycosides on column:- (i)	(ii)	Peak[a] numbers	Approx. relative molar props.
2,3,4,6-Me_4-galactose	1.67	1.67	1	1
2,4,6-Me_3-galactose	3.65	3.04	2	12
	4.10	3.48	3	
2,3,4-Me_3-galactose	6.22	5.31	4	Trace
2,6-Me_2-galactose	8.5	6.1	5	3
		7.6	6	
		8.7	7	
2,4-Me_2-galactose	13.8	9.9	8	1
	16.2	11.4	9	

[a] Peak numbers refer to those in Figure 14.

the mixture of methyl glycosides by paper chromatography in solvents (c) and (f) showed the presence of those O-methyl sugars already characterised by g.l.c. of their methyl glycosides (Table 9).

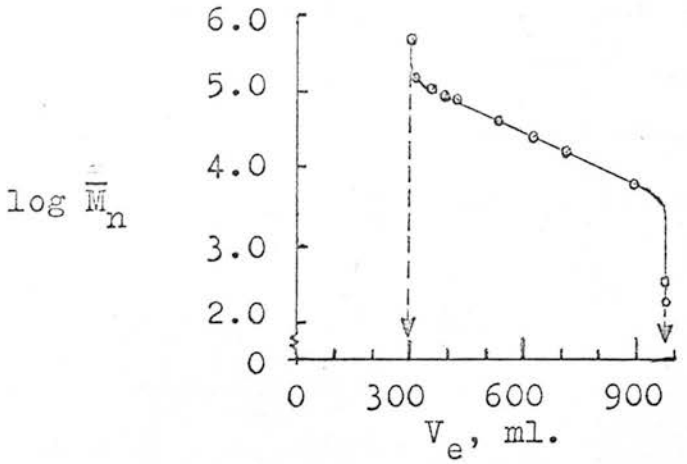


Figure 15. Plot of elution volume, V_e, against log $\overline{M}_n$ for dextran fractions of known $\overline{M}_n$ values. "Bio-Gel P300" column (50 x 4.8 cm.): elution with M-sodium chloride. The arrows correspond with those on Figure 16.

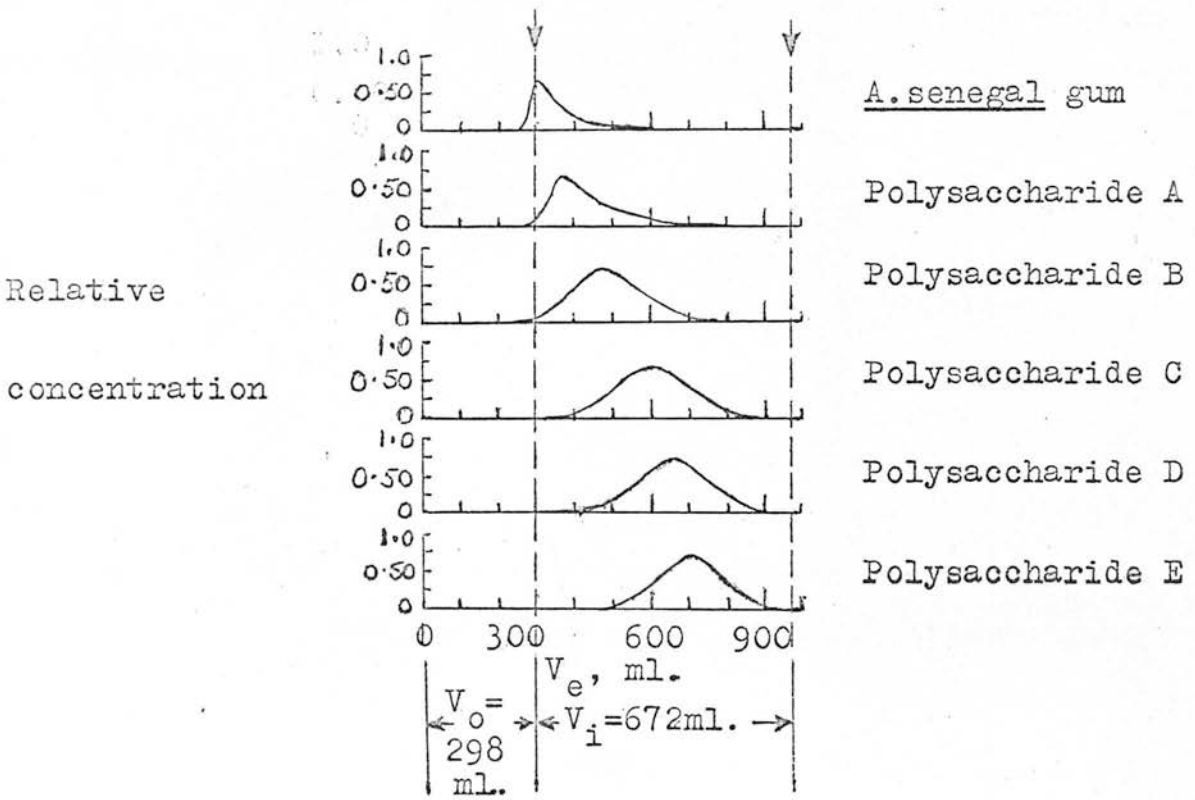


Figure 16. Elution patterns for Acacia senegal gum and for polysaccharides A - E. "Bio-Gel P300" column (50 x 4.8 cm.): elution with M-sodium chloride.

Molecular-sieve chromatography of polysaccharides A - E.- Figure 15 shows a calibration plot of V_e against log $\bar{M}_n$ obtained with dextran fractions of known $\bar{M}_n$. Figure 16 shows the elution patterns obtained for A.senegal gum and polysaccharides A - E. Table 10 gives the values found for K_d and $\bar{M}_n$; the estimated value of $\bar{M}_n$ for polysaccharide A must be regarded as approximate as a result of the asymmetric nature of its elution curve.

Table 10.

Estimation of $\bar{M}_n$ of polysaccharides A - E by molecular-sieve chromatography.

	V_e	K_d	$\bar{M}_n$
Polysaccharide A	374	0.11	96,000
Polysaccharide B	450	0.23	59,000
Polysaccharide C	590	0.43	29,000
Polysaccharide D	660	0.54	21,000
Polysaccharide E	700	0.60	17,000

Preparation of polysaccharides F and G.- Borohydride reduction of periodate-oxidised polysaccharide E (100 mg.), followed by controlled acid hydrolysis yielded polysaccharide F (70 mg., yield, 70%). The amount of formic acid released on periodate oxidation of polysaccharide E after 24 hours was

0.30 mmoles/g. During a similar degradation on polysaccharide F (50 mg.) to yield polysaccharide G (36 mg., yield, 72%), the amount of formic acid released after 24 hours was 0.35 mmoles/g.

Partial acid hydrolysis of polysaccharides F and G.- Hydrolysis of these two polysaccharides with 0.5N-sulphuric acid for 1 hour on a boiling-water bath, and paper chromatographic examination on the hydrolysate in solvent (e), indicated the presence of galactose, 3-O-β-D-galactopyranosyl-D-galactose (R_{gal} 0.52), trace amounts of 6-O-β-D-galactopyranosyl-D-galactose (R_{gal} 0.36), and the β1,3-linked galactotriose (R_{gal} 0.23).

Preparation and partial acid hydrolysis of polysaccharide E'.- A sample (60 g.) of gum was subjected to five successive Smith degradations, without isolation of the intermediate polysaccharides, to obtain polysaccharide E' (2 g.). This polysaccharide was hydrolysed with 0.5N-sulphuric acid (500 ml.) for 1 hour on a boiling-water bath. The cooled solution was neutralised (barium hydroxide and barium carbonate), filtered, deionised [Amberlite resin IR-120 (H)], and concentrated. The syrup was chromatographed in solvent (a) on Whatman No.3MM papers, and two pure disaccharides and one trisaccharide were isolated.

Fraction 1 (11.2 mg.) had $[\alpha]_D$ +35° (c, 0.22) and yielded galactose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 0.31 in solvent (a), 0.20 in solvent (b), and 0.37 in solvent (e)] as an authentic sample of 6-O-β-D-galactopyranosyl-D-galactose. A portion (8 mg.) was

methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra (i, T 1.68; ii, T 1.67) and 2,3,4-tri-O-methyl-D-galactose (i, T 6.33; ii, T 5.27).

Fraction 2 (60 mg.) had $[\alpha]_D$ +61° (c, 1.20) and yielded galactose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 0.49 in solvent (a), 0.27 in solvent (b), and 0.53 in solvent (e)] as an authentic sample of 3-O-β-D-galactopyranosyl-D-galactose. This disaccharide was crystallised from aqueous acetone to give needles of 3-O-β-D-galactopyranosyl-D-galactose monohydrate, which had m.p. 156° - 159° [Lit., m.p. 159° - 160°, Bailey (1965)].

Fraction 3 (50 mg.) had $[\alpha]_D$ +52° (c, 1.00) and yielded galactose and the β1,3-galactobiose on partial acid hydrolysis. The trisaccharide was crystallised from aqueous ethanol, m.p. 238° - 242° (decomp.) [Lit., m.p. 240° - 245°, Bailey (1965)] and had R_{gal} values of 0.22 in solvent (a) and 0.24 in solvent (e).

After prolonged hydrolysis (24 hours) of polysaccharide E′ (15 mg.) with 0.5N-sulphuric acid (5 ml.) on a boiling-water bath, the β1,6-galactobiose could not be detected by paper chromatography in solvents (a) and (e).

(I wish to thank Dr. G.O. Aspinall and Dr. D.A. Rees for their provision of methyl glycosides of authentic O-methyl sugars.

I am also grateful to Professor J.K.N. Jones, F.R.S. and Professor R.L. Whistler for gifts of the β1,4- and α1,4-galactobioses, respectively).

3.3) Discussion.

Any investigation of a plant gum polysaccharide must attempt to assess the nature of its heterogeneity. Chromatography on DEAE-cellulose indicated that there were no sharp discontinuities in the properties of the molecular species of a sample of gum arabic investigated by Jermyn (1962); in contrast, however, Lewis & Smith (1957) reported that their sample of A.senegal gum was electrophoretically "heterogeneous" in 2N-sodium hydroxide solution on glass-fibre paper. Presumably these authors observed more than one component on their electrophoretograms. No evidence for more than one band was obtained when the present sample was examined by electrophoresis in 2N-sodium hydroxide solution on glass-fibre paper. Furthermore, the sample migrated as a single band on electrophoresis in 0.1M-ammonium carbonate buffer (pH, 8.9) and 0.1M-acetate buffer (pH, 4.8) on both Whatman No.1 paper and cellulose acetate film; it was also shown by Smith (1966) to be eluted as a single peak from a DEAE-cellulose column [gradient elution with 0 - 0.5M-phosphate buffer (pH, 6.0)], and to give a smooth fractional precipitation curve on graded addition of acidified ethanol.

Fractional precipitation of gum arabic with propan-2-ol (Heidelberger, Adams & Dische, 1956) and acetone (Van Beek, 1958)

has been reported. Van Beek (1958) has also suggested that there is a correlation between the limiting viscosity numbers of fractions precipitated by acetone and their content of divalent cations. Since prolonged contact of gum with organic solvents leads to insolubility difficulties, the possibility of using a salt as a fractional precipitant was examined; sodium sulphate proved successful in this respect and four fractions were obtained from A.senegal gum. As in any fractional precipitation procedure, the number of fractions isolated is arbitrary and is governed by the amount of starting material available, and the amount of material required for the analysis necessary to characterise the fractions. The analytical results are summarised in Table 1. Although the values for the specific rotations, and the amounts of formic acid released on periodate oxidation show little variation, significant differences in the proportions of galactose to arabinose indicate that the gum is chemically heterogeneous. Previous evidence for chemical heterogeneity in a sample of commercial gum arabic has been obtained from immunological experiments carried out by Heidelberger & Adams (1956); a small fraction of gum precipitated by Type II antipneumococcal horse serum was depleted in rhamnose.

Viscosity and light-scattering measurements on A.senegal gum, and on sodium sulphate fractions I, II, and III, were made in M-sodium chloride solution; this molarity of sodium chloride is sufficient to annul (Rahman, 1966) the electroviscous effect

(Hartley, 1948); in M-sodium chloride solution, the gum may be considered to behave like a neutral polymer. Careful electrodialysis of A.senegal gum, and the sodium sulphate fractions, eliminated the possibility (cf. Van Beek, 1958) of traces of di- and poly- valent cations causing aggregation. Aggregation of gum molecules by protein is also unlikely to occur in M-sodium chloride solution, since coacervates are broken down on addition of simple electrolytes (Bungenberg de Jong, 1949). The differences in the limiting viscosity numbers of the fractions must then reflect differences in the average size and/or shape of the gum molecules. Light-scattering experiments show that there is a variation in weight-average molecular-weight. Rahman (1966) and Anderson et al. (1966) have noted a similar variation in weight-average molecular-weight for another sample of A.senegal gum and sodium sulphate fractions derived from it. The value of 600,000 for the weight-average molecular-weight of A.senegal gum is in agreement with the value of 580,000 reported by Deb & Mukherjee (1962) and by Anderson et al. (1966) for other samples of A.senegal gum. Deb & Mukherjee (1962) obtained their value for gum arabic in a solution, 0.02M with respect to a mixture of calcium, magnesium, and potassium chlorides. Aggregation through cross-linking by calcium and magnesium ions does not therefore appear to be important in A.senegal gum solutions (cf. Van Beek, 1958). Even although, it is desirable to avoid addition of di- and poly-valent cations to gum solutions prior to any physico-chemical

measurements. The light-scattering measurements made by Veis & Eggenberger (1954) and Deb & Mukherjee (1962) have already been criticised (Anderson et al., 1966) for not employing a sufficiently high molarity of simple electrolyte to annul the electroviscous effect.

The values for the intermolecular interaction constant, B', in the equation,

$$\frac{\eta}{c}\,sp = B'c + [\eta]$$

increase with increasing weight-average molecular-weight; it is reasonable to expect such interactions to be more important for larger molecules. The values of 0.54 for α and 1.3×10^{-2} for K' (cf. Rahman, 1966) in the modified Staudinger (1932) equation must be viewed with a considerable degree of caution. Clearly, more experiments are required to establish if these values will be generally applicable to all A.senegal gum samples.

Molecular-sieve chromatography (Hjertén & Mosbach, 1962; Anderson & Stoddart, 1966b) or gel filtration (Porath & Flodin, 1959) has been carried out on a number of media, including agar (Polson, 1961), cross-linked dextran gels (Porath & Flodin, 1959) [commercially available as "Sephadex"] polyacrylamide gels (Hjérten, 1962) [commercially available as "Bio-Gel"] and porous glass (Haller, 1965) [commercially available as "Bio-Glas"]. The possibility of estimating molecular-weights of substances from their behaviour on molecular-sieve columns was first suggested by Lathe & Ruthven (1956). Andrews (1962, 1964, 1965) has realised this possibility with proteins and

has reported an approximately linear relationship between the logarithms of molecular-weights of proteins and their elution volumes, V_e, from an agar column (Andrews, 1962) and from columns of various grades of "Sephadex" (Andrews, 1964, 1965). Granath & Flodin (1961) have noted a similar relationship for the behaviour of dextran fractions on columns of cross-linked dextrans, and Andrews & Roberts (1962) have suggested the possibility of applying molecular-sieve chromatography to molecular-weight estimations on polysaccharides. The commercially available polyacrylamide gels ("Bio-Gel") offer an opportunity for studying the molecular-sieve chromatographic behaviour of polysaccharides on columns of non-carbohydrate material (Anderson et al., 1965; Anderson & Stoddart, 1966c; Anderson, Hirst & Stoddart, 1966).

Molecular-sieve chromatography has been the subject of several reviews (Tiselius, Porath & Albertsson, 1963; Determann, 1964; Ogston, 1966; Andrews, 1966; Anderson & Stoddart, 1966b). It is a form of partition chromatography, which utilises the distribution behaviour of a solute between solvent phases in two physically distinguishable environments. If the solvent is allowed to flow through a column packed with porous particles, the proportion of the solvent imbibed within the pores constitutes the stationary phase and the proportion of the solvent occupying the void space between the porous particles constitutes the mobile phase; the remainder of the solvent molecules are so strongly associated with the porous particles that they are

inaccessible to solute molecules. Therefore, it follows that, when solvent is flowing through a column of porous particles, the total volume, V_t, of the column is given by the equation

$$V_t = V + V_o + V_i \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where V is the volume of the solvated porous particles, V_o is the void volume (or volume of the mobile phase) and V_i is the internal volume (or volume of the stationary phase). The behaviour of a solute passing through such a column is described by a distribution coefficient, K_d, which is defined (Gelotte, 1960) as the fraction of the internal volume, V_i, that is accessible to the solute i.e.

$$K_d = \frac{V_e - V_o}{V_i} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where V_e is the elution volume of the solute. For large molecules, which cannot enter the stationary phase and are confined to the mobile phase, $K_d = 0$. For small molecules, which can distribute themselves equally between the two phases, $K_d = 1$. Molecular-sieving occurs for molecules, which obey the condition $0 < K_d < 1$.

Figure 10 shows that for dextran fractions of known number-average molecular-weight, $\bar{M}_n$, on a "Bio-Gel P300" column, the empirical relationship between log $\bar{M}_n$ and V_e is linear for values of $\bar{M}_n$ between 5,000 and 125,000. The observation that the exclusion limit is considerably less for dextrans than the value of 300,000 claimed by the manufacturers for proteins is

not surprising; Andrews (1964) has pointed out that dextran molecules in solution have more expanded structures than protein molecules of the same molecular-weight.

The estimated values of $\bar{M}_n$ for sodium sulphate fractions III and IV from A.senegal gum are in agreement with those determined by osmometry (see Table 4). Since a value of ca. 250,000, obtained by osmometry, has been reported by Oakley (1935, 1936, 1937) for the $\bar{M}_n$ of gum arabic, the elution of A.senegal gum near the exclusion limit of the "Bio-Gel P300" column is to be expected. Some "trailing" of the elution peak indicates the presence of some low molecular-weight material; this observation is confirmed by the elution patterns and elution volumes obtained for sodium sulphate fractions III and IV. Assuming a value of 250,000 for the $\bar{M}_n$ of A.senegal gum, the molecular-weight distribution, as measured by the ratio, $\bar{M}_w/\bar{M}_n$, has a value of 2.4. A value of this magnitude for this ratio implies that the molecules, which comprise A.senegal gum, exhibit a wide molecular-weight distribution.

The value of 4,800 for the $\bar{M}_n$ of autohydrolysed gum is in agreement with the value of 4,400 determined by end-group analysis; these values will be compared later on in this discussion.

The use of molecular-sieve chromatography in the estimation of molecular size within the polysaccharide group of macromolecules is still in its infancy. Clearly, more experiments with polyacrylamide gel ("Bio-Gel") and porous glass ("Bio-Glas") materials are necessary before the validity of their application

to molecular-weight estimations may be assessed.

It was stated at the beginning of this discussion that any investigation of a plant gum polysaccharide must attempt to assess the nature of its heterogeneity. Initially, however, three kinds of polysaccharide heterogeneity need to be distinguished. The first kind results from a mixture of polysaccharide types containing two or more structural entities e.g. starch, since it contains amylose and amylopectin. For reference to this kind of heterogeneity, the term, polydisperse, which describes polymer systems containing more than one component, may be used (cf. Greenwood & Matheson, 1956). In the second kind, the polysaccharide is heterogeneous, because it is composed of polymer molecules having the same repeating unit but with a distribution of molecular-weight e.g. amylose, since it is a linear α1,4-linked glucan with a wide range of molecular-weights. This kind of heterogeneity is almost exclusively physical; any chemical heterogeneity is a direct consequence of physical heterogeneity. For reference to this kind of heterogeneity, the term, polymolecular, which denotes a polymer having a variation in molecular-weight, may be used (cf. Greenwood & Matheson, 1956). The third kind of heterogeneity relates to polysaccharide molecules, which have a variation in monosaccharide composition and/or a variation in the mode of linking and branching of the monosaccharide units in addition to a distribution in molecular-weight. The term, heteropoly-molecular, has been used (Anderson & Stoddart, 1966c) to describe

this kind of heterogeneity.

Unfortunately, polysaccharide chemistry is a field of polymer chemistry where terminology is somewhat vague (Banks & Greenwood, 1963). The term, homogeneous, has been used indiscriminately in the literature to indicate that polysaccharides are not polydisperse, even although they are undoubtedly physically heterogeneous (i.e. have a molecular-weight distribution) and often chemically heterogeneous. In polysaccharide chemistry, the problem is not one of homogeneity; rather it is one of heterogeneity (cf. Hirst, 1961). No polysaccharide yet investigated has been shown to be composed of molecules identical in molecular-weight and molecular structure. This situation may be compared with that in protein chemistry, where it is known that some proteins are homogeneous in the sense that they are composed of identical molecules.

There is no evidence from results of investigations reported in this thesis to suggest that the present sample of A.senegal gum is polydisperse. In the absence of any strict proof of polydispersity, it may be tentatively concluded that the gum is heteropolymolecular i.e. it is comprised of a continuous spectrum of related molecular species (Norman, 1937; Hirst, 1958, 1959, 1961).

The O-methyl derivative of A.senegal gum was subjected to methanolysis and the mixture of methyl glycosides was analysed by gas-liquid partition chromatography (Bishop & Cooper, 1960; Aspinall, 1963); the methyl glycosides of 2,3,4-tri-O-methyl-L-rhamnose, 2,3,5- and 2,3,4-tri-, and 2,5-di-O-methyl-L-

arabinose, 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-O-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-O-methyl-D-glucuronic acid were identified. With the exception of 2,3,4-tri-O-methyl-D-galactose, all these O-methyl sugars were present in the methylated gum studied by Aspinall, Charlson, Hirst & Young (1963); 2,3,4-tri-O-methyl-L-arabinose, and 2,3,4- and 2,4,6-tri-O-methyl-D-galactose were not reported in the methylated gum sample investigated by Smith (1940). The identification of 2,3,4-tri-O-methyl-L-arabinose, which is proof of the presence of non-reducing L-arabinopyranose end-groups, is not unexpected, since graded acid hydrolysis of the gum has yielded 3-O-β-L-arabinopyranosyl-L-arabinose (Andrews & Jones, 1955). The identification of small amounts of 2,3,4- and 2,4,6-tri-O-methyl-D-galactose indicates that there is a small proportion of D-galactose units in the gum, which are only 6-O- and 3-O-substituted, respectively:-

....6 D-galp 1.... ....3 D-Galp 1....

The identification of 2,4,6-tri-O-methyl-D-galactose as a product from the methylated whole gum indicated that a re-examination of the methylated degraded gum (Smith, 1939b), obtained after autohydrolysis and methylation, was necessary.

Autohydrolysis of the gum, purified by electrodialysis, was carried out by heating a 2% (w/v) solution, pH 2.8, on a boiling-water bath until the optical rotation was constant

(50 hours); although these conditions were less drastic than those used by Smith (1939a), they resulted in the release of D-galactose residues in addition to the more acid-labile sugar residues, which have been previously identified (Smith, 1939a) as L-arabinose, L-rhamnose, and 3-O-α-D-galactopyranosyl-L-arabinose. Furthermore, trace amounts of aldobiouronic acids were liberated, together with acidic oligosaccharides, which were small enough to pass through cellophane dialysis tubing. Acidic material of low molecular-weight has also been obtained after autohydrolysis of the exudate gums from A.karroo (Charlson, Nunn & Stephen, 1955a) and A.cyanophylla (Charlson, Nunn & Stephen, 1955b); while this observation has led Hirst (1958) to suggest that some acidic residues may occur in acid-labile side chains, it is more likely that autohydrolysis is not nearly as selective a means of degradation as has often been supposed (cf. Smith & Montgomery, 1959). This view is supported by the fact that the autohydrolysed gum, obtained after exhaustive dialysis of the autohydrolysate, contains 2% of arabinose, in addition to uronic acid (21%) and galactose (77%). Thus, although the conditions of autohydrolysis were sufficient to break some galactopyranosidic bonds to give galactose, arabinose was not completely removed from the degraded polymer, which remained behind after dialysis (cf. O'Colla, O'Donnell & Feeley, 1962).

The significance of the methoxyl content in the autohydrolysed gum was questioned, when it was observed that the

ratio of methoxyl to uronic acid content was the same as for the whole gum. The methoxyl group is not present as the ester of D-glucuronic acid, since the methoxyl content does not decrease on attempted saponification. Methoxyl groups are now known (Smith & Montgomery, 1959) to occur commonly in plant gums in residues of 4-O-methyl-D-glucuronic acid. A careful paper chromatographic re-examination of a hydrolysate of the autohydrolysed gum resulted in the detection of a spot, which had the same mobility as 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose; the presence of this aldobiouronic acid in A.senegal gum has not been recognised by earlier investigators. It is known that other Acacia gums, which have methoxyl contents (Anderson, Cree, Herbich, Karamalla & Stoddart, 1964) contain aldobiouronic acids in which 4-O-methyl-D-glucuronic acid is present (Young, 1963; Shaw & Stephen, 1965; Anderson & Karamalla, 1966a,b; Cree, 1966). The sample of A.senegal gum used in the present study has a methoxyl content of 0.23%; this corresponds to a content of 1.5% for the anhydride of 4-O-methyl-D-glucuronic acid. The presence of this residue in A.senegal gum may account for some of the 2,3,4-tri-O-methyl-D-glucuronic acid obtained on hydrolysis of methylated gum (Smith, 1940); previously it had been thought that the small amount of the 2,3,4-tri-O-methyl-D-glucuronic acid component, obtained from the methylated gum, might arise, either from some degradation during methylation, or from a small number of non-reducing D-glucuronic acid end-groups (Smith, 1940; Smith & Montgomery, 1959); that there are

some such terminal D-glucuronic acid residues is not excluded by the present evidence.

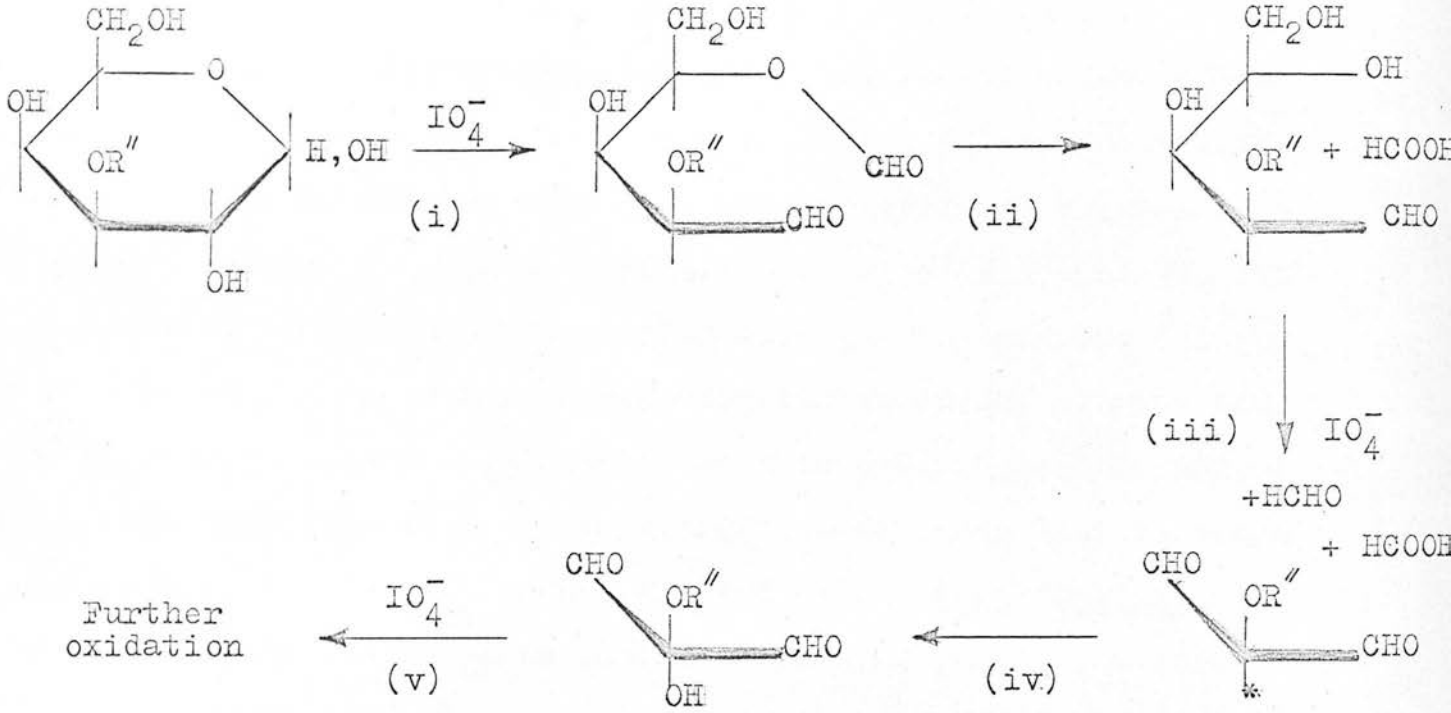
Smith (1939b) reported that hydrolysis of methylated autohydrolysed gum yielded 2,3,4,6-tetra-, 2,3,4-tri- and 2,4-di-O-methyl-D-galactose, and 2,3,4-tri-O-methyl-D-glucuronic acid in the approximate molar proportions, 1:5:3:3. A re-examination of the methylated autohydrolysed gum has, however, shown the presence of 2,3,4,6-tetra- (++), 2,4,6- (++) and 2,3,4-tri- (+++), 2,4-di- (+++), and 2-O-methyl-D-galactose (+), 2,3,4-tri-O-methyl-D-glucuronic acid (+++), and trace amounts of 2,3,5-tri-O-methyl-L-arabinose. The approximate relative molar proportions indicated in parentheses were assessed semiquantitatively from the peak areas obtained for the methyl glycosides by gas-liquid partition chromatography, and from the intensities of the spots on the paper chromatograms given by the O-methyl sugars after hydrolysis of their methyl glycosides (Aspinall & Baillie, 1963). The presence of 2-O-methyl-D-galactose is ascribed to incomplete methylation of some 3,6-di-O-substituted galactose residues at the 4-position; undermethylation, and problems arising from it, will be discussed at a later stage. Increased proportions of 2,3,4-tri-O-methyl-D-galactose in the methylated autohydrolysed gum undoubtedly arise from 1,6-linked galactose residues, which are 3-O-substituted with acid labile arabinofuranose units in the whole gum. The presence of increased proportions of 2,4,6-tri-O-methyl-D-galactose likewise suggests that some of the residues in the chains of 1,3-linked galactose units are 6-O-

substituted with acid labile arabinofuranose units in the whole gum. It follows that not all the galactose residues in the chains of β1,3-linked units carry galactose-containing side chains as had previously been accepted (Smith & Montgomery, 1959; Aspinall, Hirst & Nicolson, 1959).

Molecular-sieve chromatography of autohydrolysed gum gave an estimated value of 4,800 for the $\overline{M}_n$. Thus, during autohydrolysis, extensive degradation of the gum macromolecule [($\overline{M}_n$ ca.250,000) (Oakley, 1935, 1936, 1937)] occurs to yield a polysaccharide of relatively low molecular-weight. This degradation is much greater than can arise from removal of acid labile arabinofuranose and rhamnopyranose residues from the periphery of the molecule. A similar observation has been made by Smith & Montgomery (1959); it led them to suggest that some acid labile sugar residues might be present in the interior chains of the gum molecule; they postulated that degraded units might be interconnected by acid sensitive arabinofuranose units. If this were so, it should be possible to show that some, if not all, of this arabinose is sited at the reducing end of the degraded molecules resulting from autohydrolysis. In order to discover whether arabinose was present as the reducing end-group, the autohydrolysed gum was reduced with borohydride; the presence of galactitol, and the absence of arabinitol, in the hydrolysate of this reduced material shows that galactose occupies the reducing end-group. The 2% of arabinose in the autohydrolysed gum appears, therefore, to be sited other than at the reducing

end; in fact, trace amounts of 2,3,5-tri-O-methyl-L-arabinose, obtained from the methylated autohydrolysed gum, indicate that this arabinose is present as a small proportion of non-reducing arabinofuranose end-groups, which are not completely removed during autohydrolysis. If the extensive degradation of the macromolecule is not caused by the presence of internal labile arabinofuranosidic bonds, then certain galactosidic bonds must be unusually reactive towards very mild conditions of acid hydrolysis. There is no evidence from the methylation studies for the presence of any galactofuranose residues; this implies that certain galactopyranosidic bonds must be unusually sensitive to the mild conditions of autohydrolysis. Stephen (1963a,b) has observed that complete disruption of the macromolecules of Virgilia oroboides gum is brought about under acidic hydrolysis conditions, which, in disaccharides, would be expected to cleave furanosidic but not pyranosidic bonds; this has led him to suggest that the carboxyl groups on the uronic acid residues may play an important role in bringing about deep-seated decomposition by participating in the severing of glycosidic bonds at positions two or more sugar units removed from a uronic acid residue. However, the fact that highly branched arabinogalactans also exhibit this peculiar phenomenon (Bouveng & Lindberg, 1956; 1958; Bouveng, 1959a,b, 1961) would seem to imply that some other explanation for the unexpected lability of certain galactopyranosidic bonds in polysaccharides of the arabinogalactan and substituted arabinogalactan type must be sought.

Figure 17. A reaction scheme for periodate oxidation of 3-O-substituted reducing D-galactose residues at room temperature with excess of sodium metaperiodate.



(i) Malapradian oxidation.

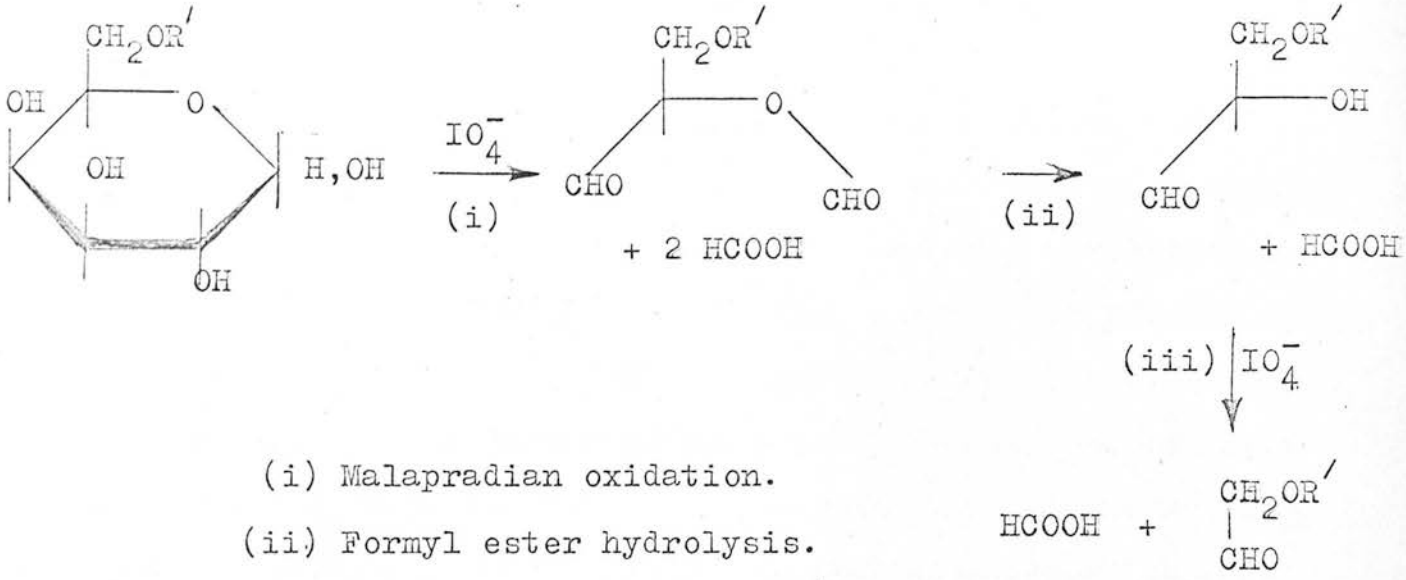
(ii) Formyl ester hydrolysis.

(iii) Malapradian oxidation.

(iv) The O-substituted malondialdehyde is susceptible to over-oxidation at the activated H atom (marked *)(Hough & Perry, 1956; Hough, Woods & Perry, 1958; Cantley, Hough & Pittet, 1959; Anderson, Hirst, Manners & Ross, 1958). The mechanism of this oxidation is uncertain but it may involve reduction of iodate rather than periodate (Manners, 1959).

(v) Further Malapradian oxidation.

Borohydride-reduced autohydrolysed gum was assumed to produce one formaldehyde molecule per autohydrolysed gum molecule on periodate oxidation; on this basis, a value of 4,400 was calculated for the $\bar{M}_n$. Methylation evidence for the autohydrolysed gum suggests that the reducing galactose units could be 3-O-substituted and/or 6-O-substituted and/or 3,6-di-O-substituted. A reaction scheme for periodate oxidation of 3-O-substituted galactose residues at room temperature with excess of sodium metaperiodate is shown in Figure 17. Production of formaldehyde at stage (iii) is dependent on the successful hydrolysis of the formyl ester [stage (ii)]; this hydrolysis usually occurs during oxidation with a limited excess of sodium metaperiodate at room temperature (Anderson, Hirst, Manners & Ross, 1958). Since no formaldehyde is released on periodate oxidation of autohydrolysed gum, either the ester hydrolysis does not occur, or all the reducing galactose units are also 6-O-substituted; if all the reducing galactose units are not 6-O-substituted, and if ester hydrolysis does not occur, then the value of 4,400 for the $\bar{M}_n$, calculated from the formaldehyde released after periodate oxidation of borohydride-reduced autohydrolysed gum, will be a low estimate (cf. the value of 4,800, obtained by molecular-sieve chromatography). A reaction scheme for periodate oxidation of 6-O-substituted reducing galactose residues at room temperature with excess of sodium metaperiodate is shown in Figure 18. No formaldehyde would be released by such residues, and moreover, they are not susceptible to over-oxidation



(i) Malapradian oxidation.

(ii) Formyl ester hydrolysis.

(iii) Malapradian oxidation.

Figure 18. A reaction scheme for periodate oxidation of 6-O-substituted reducing galactose residues at room temperature with excess of sodium metaperiodate.

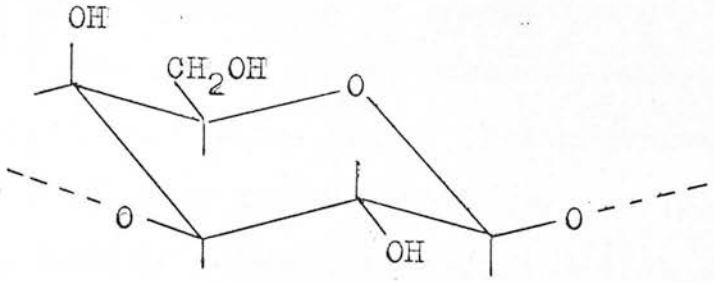


Figure 20. A β1,3-linked D-galactose residue in the C 1 conformation.

(Hough & Perry, 1956). There is evidence for a proportion of 3-O-substitution and/or 3,6-di-O-substitution at reducing galactose units: a controlled Smith degradation (cf. Smith & Spriestersbach, 1955) carried out at 2°C gives a product, which on acid hydrolysis, yields arabinitol. The formyl ester group is stable at 2°C (Anderson et al., 1958), and so arrests further Malapradian oxidation. The series of reactions proposed (cf. Smith & Montgomery, 1959) in Figure 19 accounts for the appearance of this sugar alcohol; its identification is evidence that at least some of the reducing galactose residues are substituted at C-3.

Smith & Spriestersbach (1955) reported that hydrolysis of methylated Smith-degraded autohydrolysed gum yielded only 2,3,4,6-tetra- and 2,4,6-tri-O-methyl-D-galactose. A re-examination of this methylated product has, however, shown the presence of 2,3,4,6-tetra (+), 2,4,6- (++++) and 2,3,4-tri- (a trace), 2,6- (+) and 2,4-di (+/2), and 2-O-methyl-D-galactose (+/2). The presence of 2,6-di and 2-O-methyl-D-galactose probably result from incomplete methylation. It is surprising that Smith & Spriestersbach (1955) and Smith & Montgomery (1959) did not report any difficulties arising from incomplete methylation, since polysaccharides of the arabinogalactan type that have a high proportion of β1,3-linked galactose units, are notoriously difficult to methylate (Bouveng & Lindberg, 1956; Bouveng & Lindberg, 1958; Aspinall, Hirst & Ramstad, 1958; Bouveng 1959a,b, 1961; Adams, 1960; Jones & Reid, 1963; Aspinall & Fairweather, 1965;

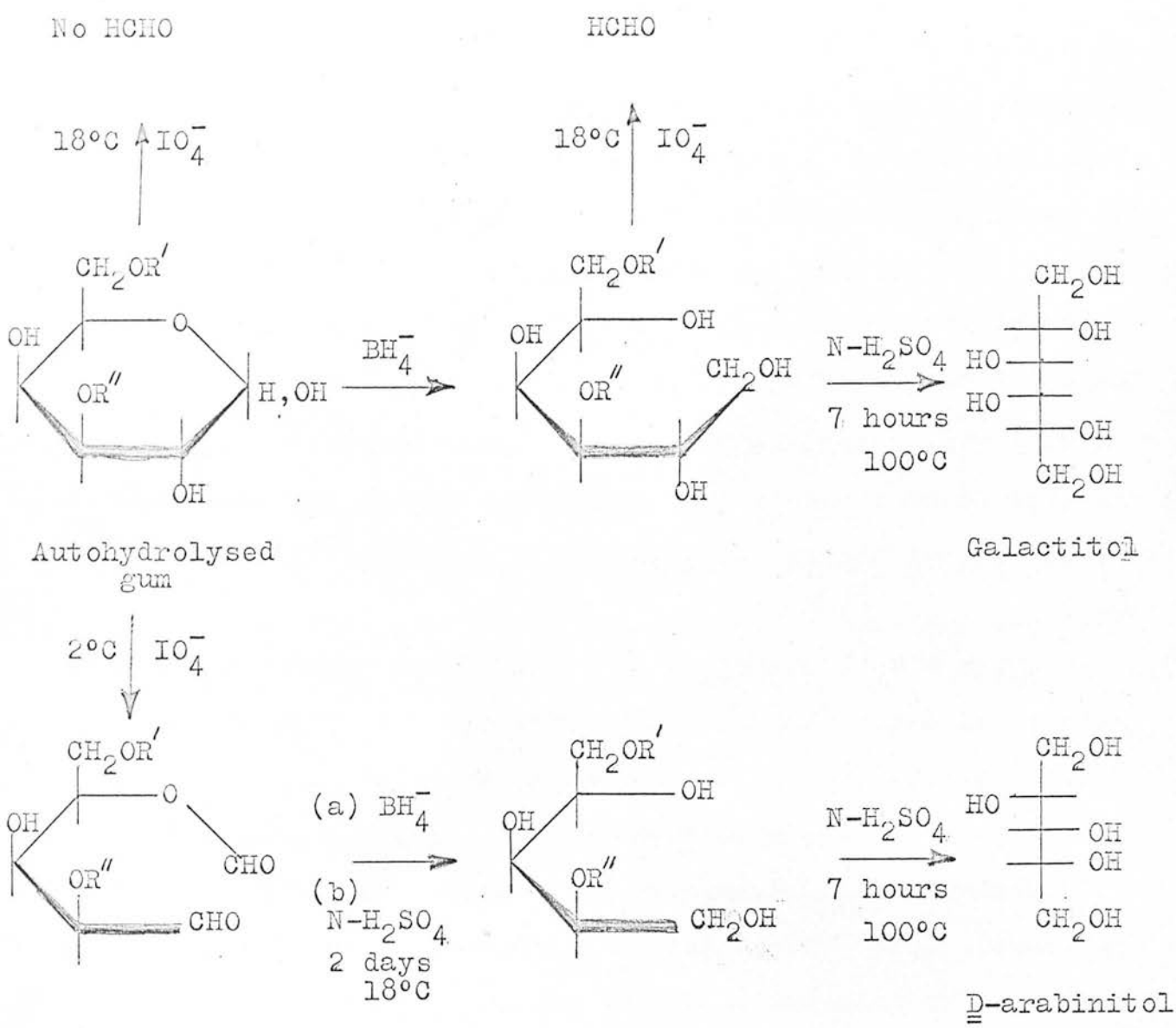


Figure 19. Scheme of reactions carried out on autohydrolysed gum if the reducing D-galactose residue is 3,6-di-O-substituted.

Wolfrom & Patin, 1965). If β1,3-linked D-galactose residues in polysaccharides of the arabinogalactan type assume the stable C1 conformation as shown in Figure 20, then the hydroxyl group at C-4 will be axial and so presumably less reactive towards methylating reagents (Bouveng & Lindberg, 1958; Aspinall & Fairweather, 1965). The presence of 2,4-di-O-methyl-D-galactose indicates some degree of branching in the Smith-degraded product; the identification of trace amounts of 2,3,4-tri-O-methyl-D-galactose indicates the presence of some 1,6-linkages other than at branch-points. While the presence of a high proportion of 2,4,6-tri-O-methyl-D-galactose is evidence for chains of 1,3-linked D-galactose units in the autohydrolysed gum, it does not necessarily follow (cf. Smith & Montgomery, 1959) that there is a "main chain" in the whole gum and that all the linkages in this "main chain" are of the 1,3-type.

A sample of the whole gum was subjected to successive degradations using the procedure, devised by Goldstein, Hay, Lewis & Smith (1959), involving periodate oxidation, borohydride reduction and controlled acid hydrolysis. The first periodate oxidation was stopped after 48 hours; after borohydride reduction, and hydrolysis of the acetal linkages with N-sulphuric acid for 2 days at room temperature, polysaccharide A was isolated. This contained galactose (69%), arabinose (27%), and glucuronic acid (4%); acid hydrolysis yielded a small amount of 6-O-(β-D-glucopyranosyluronic acid)-D-galactose. Thus, the first Smith degradation removed all the rhamnose and 4-O-methyl-

glucuronic acid residues, together with some galactose, arabinose, and glucuronic acid units. Non-reducing end-group arabinose and galactose units, as well as 6-O-substituted galactose residues and 4-O-substituted glucuronic acid residues, should be cleaved during the first Smith degradation. Methylation evidence for the whole gum indicates that there are some non-reducing end-group galactopyranose, arabinopyranose, and arabinofuranose residues, together with a few C-6 substituted galactopyranose units; most of the glucuronic acid residues are substituted at C-4, with very few apparently present as non-reducing end-groups. The isolation and characterisation, after deacetylation, of 4-O-α-L-rhamnopyranosyl-D-glucose from acetolysis of diborane-reduced acetylated gum established that some L-rhamnopyranose residues are glycosidically linked to C-4 of D-glucuronic acid units (Aspinall *et al.*, 1963). Complete oxidation of all glucuronic acid residues would be expected to occur during the first treatment with periodate; the incomplete oxidation observed may occur for one of several reasons. There may be some O-substitution by other sugar residues at either the C-2 and/or C-3 positions of certain glucuronic acid residues. On the other hand, there may be some 3,6-lactone formation. Butler & Cretcher (1929) have suggested (*cf.* Saverborn, 1944) that uronic acid residues in gum arabic exist to the extent of about 22% as lactone. However, the 1C conformation required for 3,6-lactone formation would be unstable, and so lactone formation is unlikely to occur. Ester formation is also

unlikely to be selective in involving hydroxyl groups on glucuronic acid residues. Finally, the possibility exists that incomplete oxidation may result from steric hindrance (Klosterman & Smith, 1952). Low reduction of periodate by 1,4-linked D-mannuronic acid and L-guluronic acid residues in alginic acid has also been reported (Drummond, Hirst & Percival, 1962). For some reason, as yet unknown, incomplete oxidation of 1,4-linked uronic acid residues in polysaccharides may be the rule rather than the exception.

There is an additional problem in carrying out Smith degradations on acidic polysaccharides. Difficulty is encountered when attempting to hydrolyse acetal linkages involving acidic fragments, since they tend not to be cleaved by cold dilute acid (Aspinall, Bhavanandan & Christensen, 1965; Bouveng, 1965). Such fragments should, however, be removed during subsequent Smith degradations, since glucuronic acid residues are glycosidically linked to the C-6 position of galactose residues, which are also usually 3-O-substituted with short side chains containing galactopyranose, arabinopyranose, and arabinofuranose residues. Removal of these side chains by successive Smith degradations will leave those galactose residues carrying acidic fragments on C-6 vulnerable to attack by periodate; any persistent acidic fragments will, therefore, be eliminated subsequently. Over-oxidation will be mentioned at a later stage in this discussion.

On methanolysis, the O-methyl derivative of polysaccharide A

gave the methyl glycosides of 2,3,5-tri- and 2,5-di-O-methyl-L-arabinose, 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,6- and 2,4-di-O-methyl-D-galactose, and 2,3,4-tri-O-methyl-D-glucuronic acid. A methylation study by Dillon et al. (1954) on the polysaccharide obtained after one Barry degradation gave, as hydrolytic cleavage fragments, 2,3,5- and 2,3,4-tri-, and 2,5- and 2,3-di-O-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-O-methyl-D-galactose. The results of the present investigation show that all the rhamnopyranose and arabinopyranose residues are removed by one Smith degradation. The presence of some 2,6-di-O-methyl-D-galactose probably results from under-methylation at the C-4 position of certain galactose residues.

A second Smith degradation yielded polysaccharide B, which contained galactose (89%) and arabinose (11%). On methanolysis, the O-methyl derivative of polysaccharide B gave the methyl glycosides of 2,3,5-tri- and 2,5-di-O-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,6- and 2,4-di-O-methyl-D-galactose. A Smith degradation of polysaccharide B gave polysaccharide C, which contained galactose (98%) and arabinose (2%). Methylation and methanolysis of this polysaccharide gave the methyl glycosides of 2,3,5-tri-O-methyl-L-arabinose and 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,6- and 2,4-di-O-methyl-D-galactose. Successive Smith degradations then gave polysaccharides D and E, which were examined by methylation and partial acid hydrolysis; on methylation and methanolysis, both polysaccharides D and E gave 2,3,4,6-tetra-, 2,4,6- and 2,3,4-

tri-, and 2,6- and 2,4-di-O-methyl-D-galactose as their methyl glycosides. The ratio of the amounts of 2,4,6-tri-O-methyl-D-galactose to 2,3,4,6-tetra- and 2,4-di-O-methyl-D-galactose, as judged from peak areas obtained for their methyl glycosides on gas-liquid partition chromatography, was greater for polysaccharide E than for D. Partial acid hydrolysis of polysaccharides D,E,F, and G afforded 3-O-β-D-galactopyranosyl-D-galactose, 6-O-β-D-galactopyranosyl-D-galactose (in small amounts), the β1,3-linked galactotriose, and higher oligosaccharides. The absence of the α or β1,4-linked galactobiose, and the fact that the degraded polysaccharide proved difficult to methylate, suggest that the small amounts of 2,6-di-O-methyl-D-galactose arise from under-methylation.

Partial acid hydrolysis of polysaccharide E′ (obtained after five successive Smith degradations in a duplicate experiment on a larger scale) led to the isolation of 6-O-β-D-galactopyranosyl-D-galactose. Proof of its structure followed from the value of the specific rotation, and methanolysis of the O-methyl derivative to give the methyl glycosides of 2,3,4,6-tetra- and 2,3,4-tri-O-methyl-D-galactose. Acid reversion of galactose gives the α1,6-galactobiose (Turton, Bebbington, Dixon & Pacsu, 1955). The 1,6-galactobiose isolated is unlikely to have arisen as a result of acid reversion, since it was not detectable on paper chromatograms after prolonged acid hydrolysis (cf. Andrews & Jones, 1955). Although acid reversion from galactose is not the source of the β1,6-galactobiose, the possibility of acid-

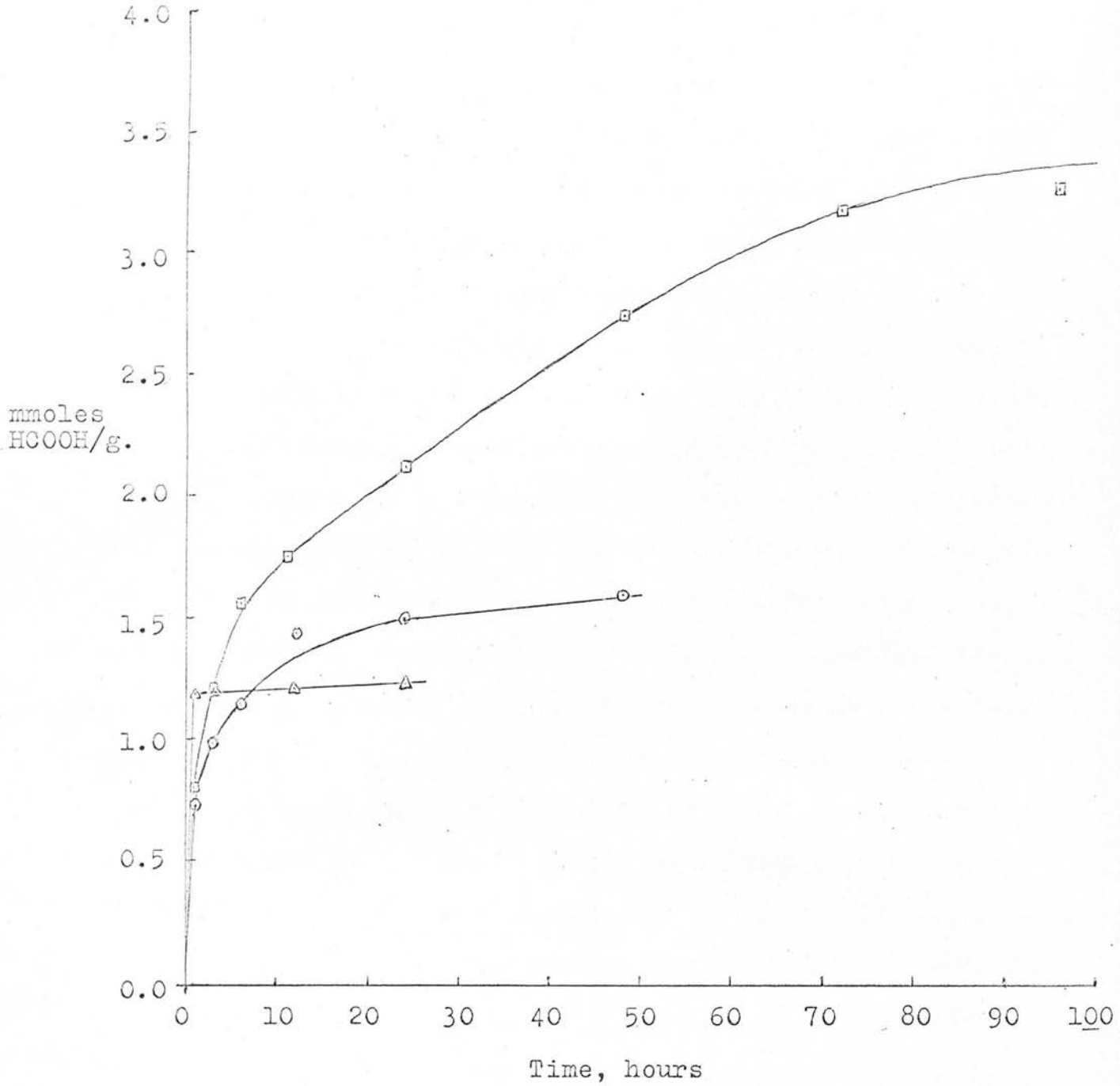


Figure 22. Formic acid released (mmoles/g.) with time (hours) on periodate oxidation of:-

(a) A. senegal gum (⊙)

(b) Polysaccharide A (⊡)

(c) Polysaccharide B (△)

catalysed transgalactosylation from β1,3-linked galactose-containing oligosaccharides cannot be ruled out (cf. Manners, 1959). Transgalactosylation, however, is unlikely to be important at the low concentrations of polysaccharide used in the partial acid hydrolysis experiments. The β1,3-linked galactobiose (Jackson & Smith, 1940b; Aspinall & Young, 1965) and galactotriose (Aspinall & Young, 1965) have been characterised previously from A.senegal gum.

Over-oxidation of polysaccharides with periodate often occurs when they contain terminal non-reducing uronic acid residues (see Figure 21). Over-oxidation is characterised by the continued slow production of acid on oxidation (Hirst & Jones, 1955). Figure 22, which presents a plot of the amount of formic acid released with time on periodate oxidation of A.senegal gum and of polysaccharides A and B, shows that polysaccharide A is susceptible to over-oxidation. The reaction mechanism proposed in Figure 23 accounts for this observation. Although Blair, Stephen & Shaw (1965) have attempted to overcome these problems in a periodate oxidation study on V.oroboides gum by first of all reducing the acetylated gum with diborane and then deacetylating, their procedure is not completely satisfactory, since it has the disadvantage that O-ethyl sugars may appear as artefacts. In the present investigation over-oxidation is not a hazard, since it assists in the elimination of acidic fragments.

Molecular-sieve chromatography of the whole gum and

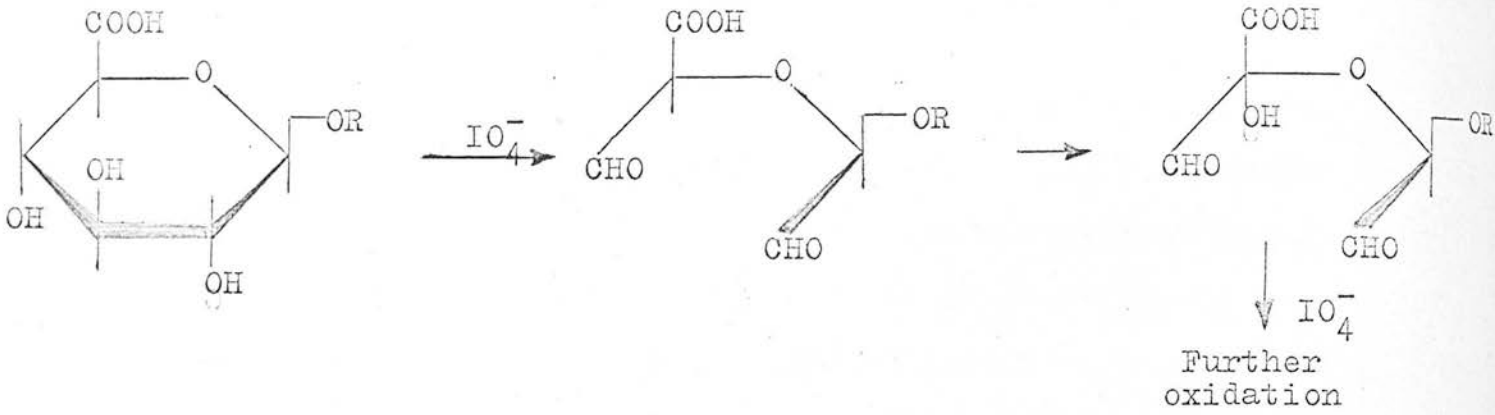


Figure 21. Reaction scheme showing the over-oxidation of terminal non-reducing D-glucuronic acid units (Huebner, Lohmar, Dimler, Moore & Link, 1945; Hirst & Jones, 1955; Smith & Montogomery, 1959).

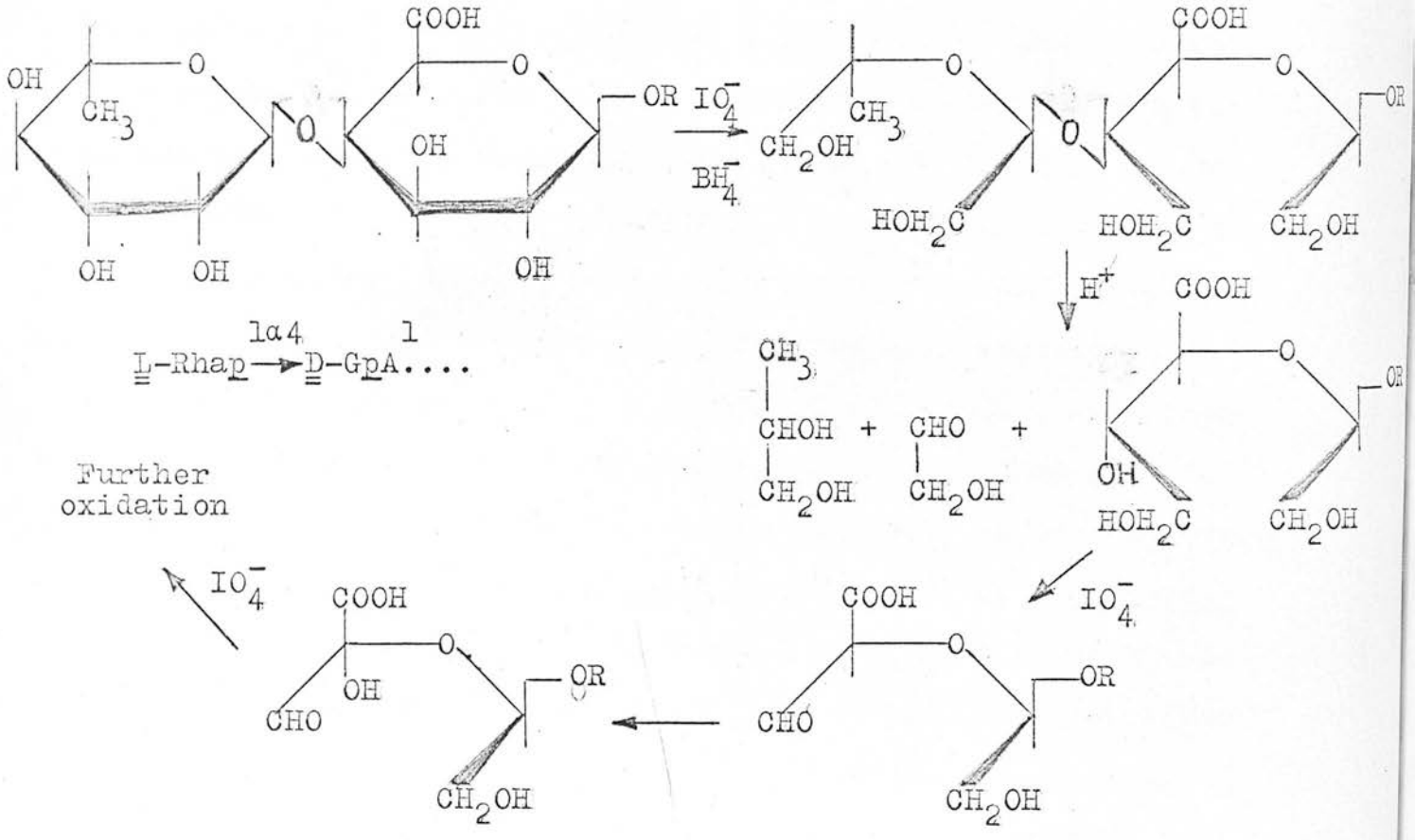
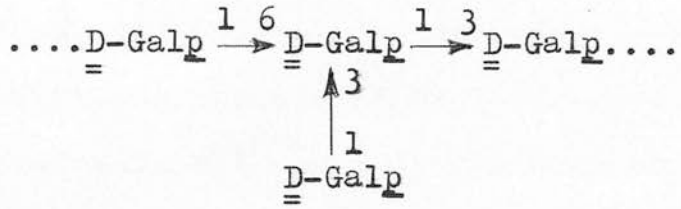


Figure 23. Reaction scheme showing the over-oxidation of D-glucuronic acid residues in A.senegal gum.

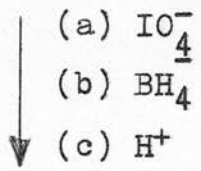
of polysaccharides A - E indicates that there is a significant drop in the $\bar{M}_n$ to ca. 96,000 for polysaccharide A after the first Smith degradation, and subsequently to 17,000 for polysaccharide E after the fifth Smith degradation. A few glycosidic bonds may be cleaved during hydrolysis of acetal linkages with cold dilute acid. Without confirmation by some more fundamental method, the values obtained by molecular-sieve chromatography must be regarded as approximate. Nevertheless, the technique has proved invaluable for following the extent of degradation during successive Smith procedures, especially when the amount of product available for analysis at each stage was limited.

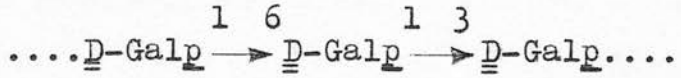
On the basis of the available evidence, several structural interpretations for polysaccharide E are possible; these must, however, take into consideration the limitations involved in applying Smith degradations in step-wise fashion to polysaccharides. It is unlikely that complete reaction is achieved at every stage in the degradation scheme. Methylation and periodate oxidation evidence indicates that polysaccharide E is not a simple linear β1,3-linked galactan. Whilst 2,4,6-tri-O-methyl-D-galactose is the most predominant O-methyl sugar obtained from the methylated polysaccharide, the identification of some 2,4-di- and 2,3,4,6-tetra-O-methyl-D-galactose indicate occasional branching at the C-3 and C-6 positions. The presence of small amounts of 2,3,4-tri-O-methyl-D-galactose in the cleavage fragments obtained from methylated polysaccharide E indicates

that there are a few D-galactose residues which are only 6-O-substituted. These could arise from Smith degradation of fragments of the following type in polysaccharide D:-



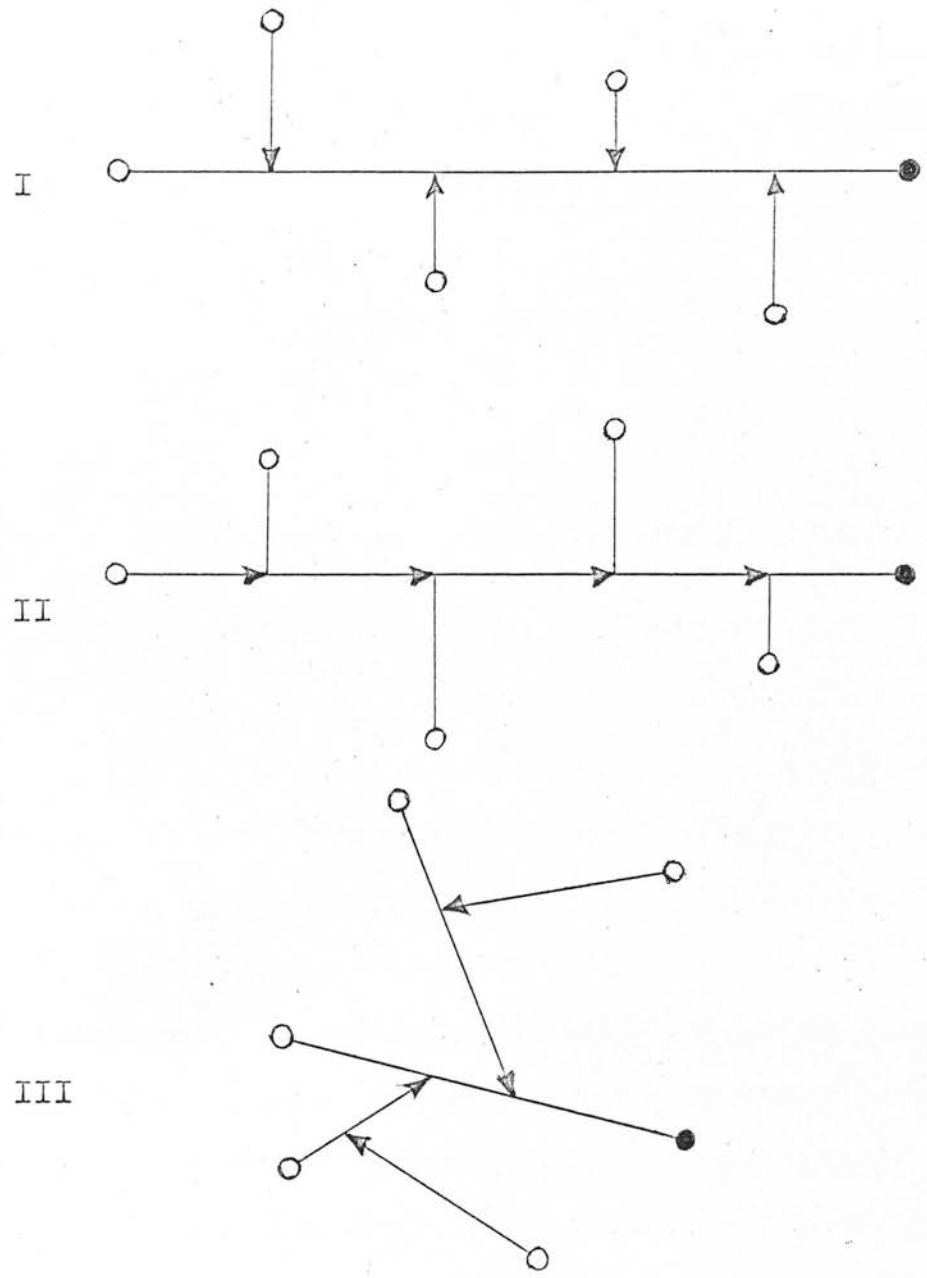
Structural fragment in polysaccharide D.





Structural fragment in polysaccharide E.

The amount of formic acid released by polysaccharide E also indicates some degree of branching; a number-average chain-length, $\overline{C}_n$, of about 21 galactose residues may be calculated from the amount of formic acid released. The presence of small amounts of the β1,6-galactobiose in the partial acid hydrolysate of polysaccharide E is further support for a branched structure; moreover, this disaccharide is also present in the partial acid hydrolysates of polysaccharides F and G. If there is a "main chain" of β1,3-linked D-galactose units in polysaccharide E, it must carry short side chains of β1,3-linked D-galactose residues; the branch points for these side chains would be



——— = chains of β1,3-linked D-Galp residues.

↓ = β1,6-linkage at branch point.

● = reducing end-group.

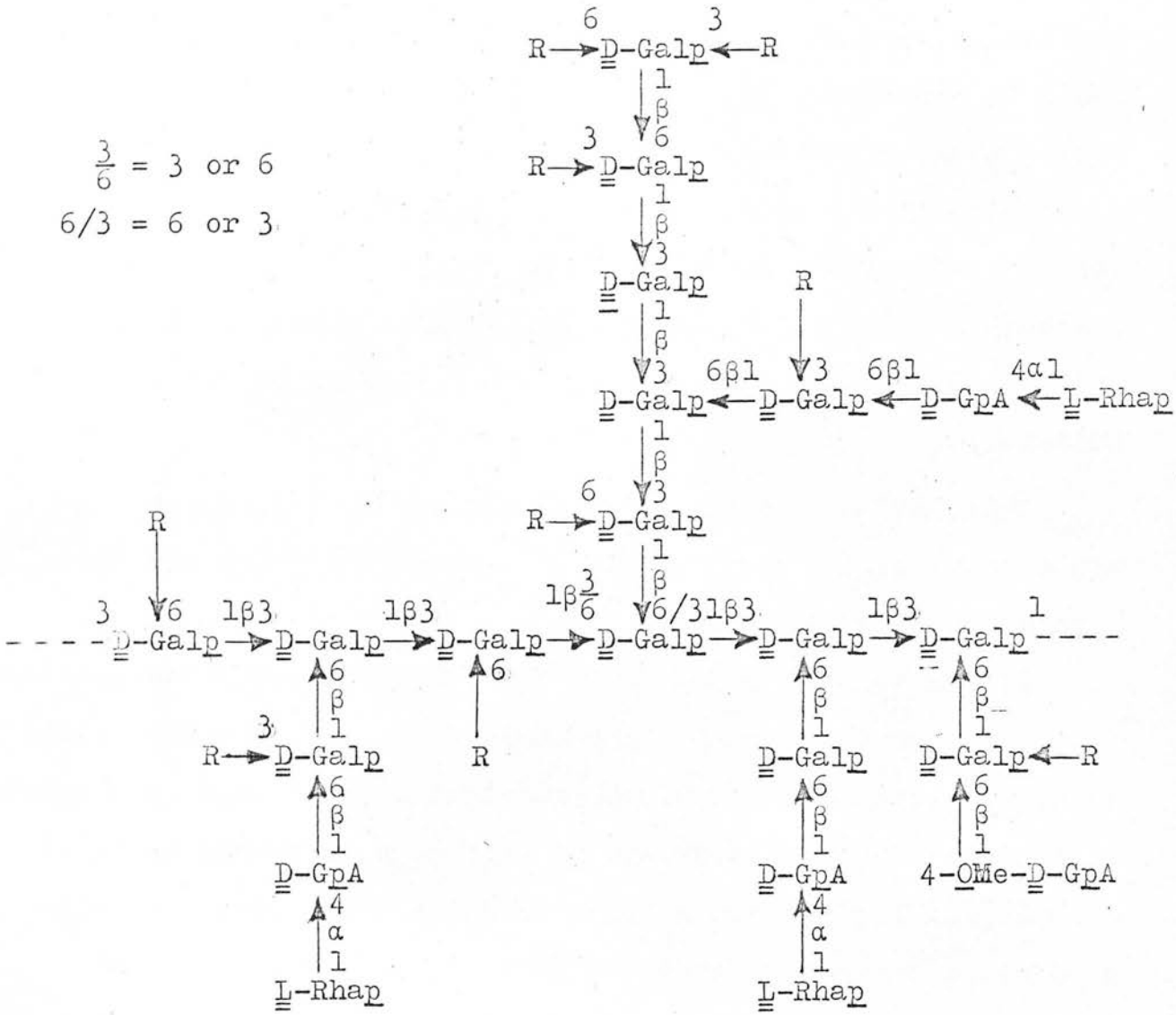
○ = terminal non-reducing group.

Figure 24. Diagrammatic representation of some of the possible structures for the periodate-resistant galactan framework (polysaccharide E) of Acacia senegal gum.

through β1,6-linkages (Figure 24, I). Alternatively, there could be occasional β1,6-linkages along a "main chain" of β1,3-linked D-galactose units; in this case, the branch points for short side chains would be through β1,3-linkages to the β1,6-linked residues in the "main chain" (Figure 24, II). A randomly branched structure of β1,3-linked chains with β1,6 branch points would also satisfy the available structural evidence.

Thus, although it is widely accepted that A.senegal gum has a "main chain" or "backbone," unequivocal proof for this hypothesis is still lacking.

It has been stated already that the polysaccharide entities that comprise the gum are most probably polymer systems which have, in addition to a molecular-weight distribution, a variation in monosaccharide composition as well as a distribution in the mode of linking and branching of certain monosaccharide units. A possible structural fragment from the internal chains of one of these entities is represented by the polysaccharide array shown in Figure 25 and by the photograph of a model shown on Plate I. At present, any of the three galactan frameworks shown in Figure 24 may be considered to form the basis for fragments of this general type. If gum molecules are based on galactan frameworks of a comb-like type (Figure 24, I and II), it is permissible to use the term, average repeating unit, in reference to the polysaccharide array (Figure 25). If, however, the galactan frameworks are randomly branched structures of



R represents D-galactopyranose-, L-arabinopyranose-, and L-arabinofuranose- containing side chains. These side chains may be up to four units long and contain ....L-Araf.... residues. They may be terminated by L-Araf...., L-Arap→L-Araf...., or D-Galp→L-Araf.... residues.

Figure 25. Possible structural fragment in Acacia senegal gum.

β1,3-linked galactose chains with β1,6 branch points (Figure 24, III), the concept of an average repeating unit is not strictly applicable, since it infers that there is a "main chain" along which the average repeating unit is repeated. As the precise nature of the galactan framework is unknown, the polysaccharide array shown in Figure 25 is recommended as a possible structural fragment rather than as an average repeating unit. This structural fragment is not unique; a large number of other possible structural fragments could be proposed.

The implication of the present structural study on A.senegal gum is that the polysaccharide entities which comprise the gum are more highly branched, and consequently more globular in shape, than has previously been recognised. There is some evidence from physical measurements, and particularly from viscometry, to support this view. The gum from A.senegal is almost unique amongst commercially important gums in forming water-soluble solutions over a very wide range of concentrations. Moreover, the gum has a small limiting viscosity number (ca. 12 - 20 ml./g., Rahman, 1966) and its solutions are of relatively low viscosity (Glicksman & Schachat, 1959) when compared, on an equal concentration basis, with polysaccharides known to have substantially linear structures and approximately the same number-average molecular-weights (e.g. locust bean gum and guaran). If the polysaccharide molecules are in the form of "stiff-coils," as has been suggested by Veis & Eggenberger (1954), these should unfold in solution as a result of inter-coulombic

repulsions between ionised carboxyl groups, to give elongated entities which would be expected by virtue of their rod-shaped nature to form viscous solutions and exhibit non-Newtonian behaviour. Recently, however, Warburton (1966) has observed that a 5% gum solution shows ideal Newtonian flow, up to a shear rate of 100 reciprocal seconds, in a Couette concentric cylinder viscometer. This has led him to suggest that, in solution, the gum gives "a suspension of non-entangling spheroids." Although Smith & Montgomery (1959) have noted that a 30% gum solution showed no structure viscosity (i.e. behaved as a Newtonian solution), Ostwald, Auerbach, Feldmann, Trakas & Malss (1934) found that structure viscosity occurs in 45% gum solutions at 20°C when pressures are below 10 cm. of water. The appearance of structure viscosity at high concentrations probably results from aggregation of macromolecules and so is not a reflection of molecular shape. In contrast to this evidence for a more globular and possibly more spheroidal model for A.senegal gum molecules, Saverborn (1945) (cf. Greenwood, 1952) has interpreted ultracentrifuge and flow birefringence measurements in terms of elongated particles. Clearly, more physico-chemical measurements on A.senegal gum solutions are required to resolve these discrepancies.

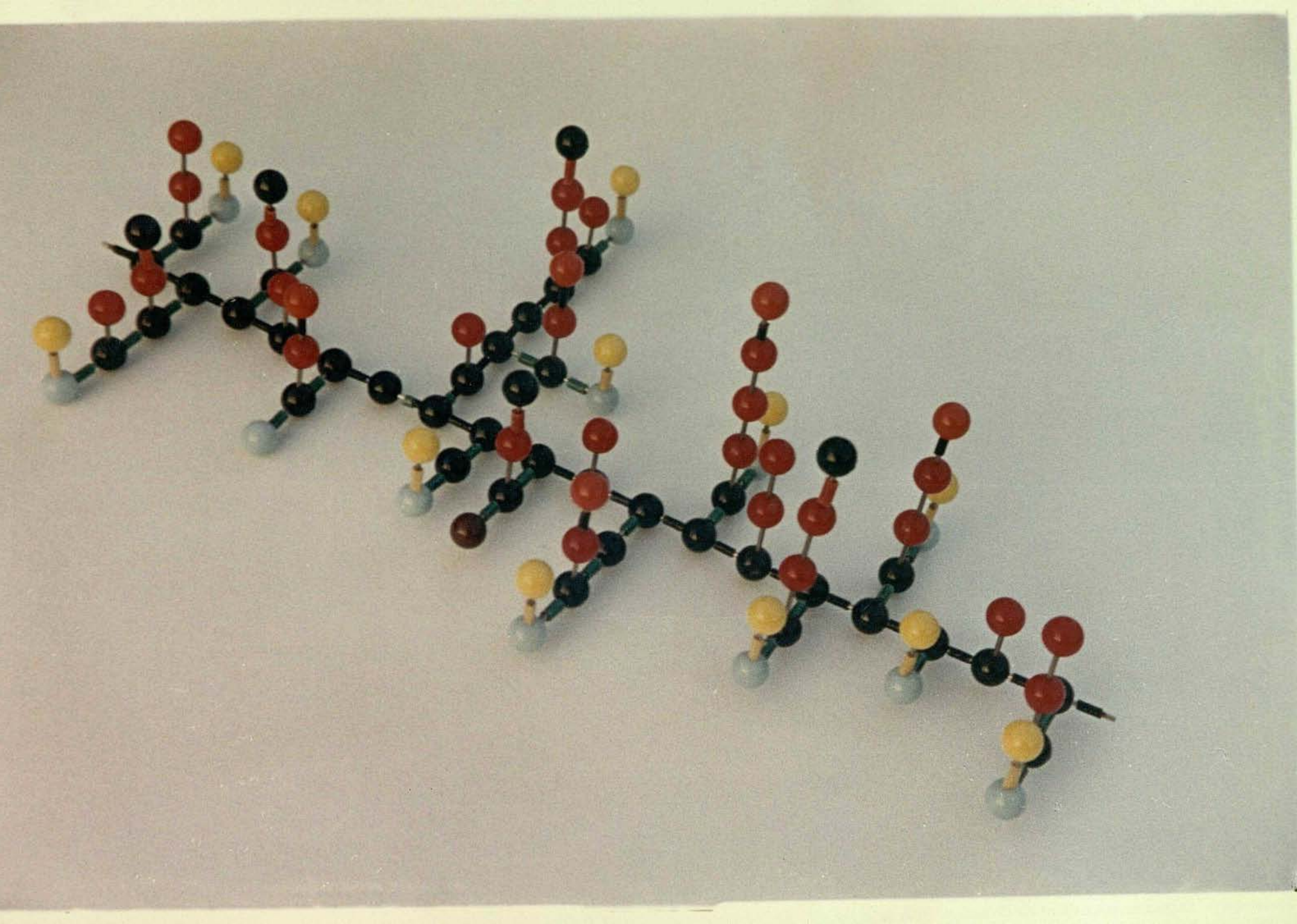


Plate I. This plate shows a colour photograph of a model, which represents the author's concept of how 100 sugar residues may be linked together in a structural fragment from a molecule of A.senegal gum. Each ball represents a sugar residue. The model portrays one possible assembly of sugar residues.

Key

Balls	Sugar residues	Bonds	Linkages
Black	D-Galp	Black	β1,3
Blue	D-GpA	Green	β1,6
Brown	4-OMe-D-GpA	Yellow	α1,4
Yellow	L-Rhap	Red	α1,3
Orange	L-Arap	No colour	1,3 unless associated with a 3-O-substituted D-Galp residue when it is 1,6
Red	L-Araf		

CHAPTER IV

SOME STRUCTURAL FEATURES OF ACACIA ARABICA GUM

4.1) Introduction

A preliminary investigation (Anderson & Karamalla, 1966a) of *Acacia arabica* gum showed that it was composed of galactose (36%), arabinose (54%), rhamnose (0.4%), and uronic acid (7.8%). The value obtained for the limiting viscosity number ($[\eta]$ = 12.5 ml./g.) was lower than that found for *A.senegal* gum (*cf.* Chapter III). The gum was also shown to have a high positive specific rotation ($[\alpha]_D$ +100°) and a high methoxyl content (OMe, 0.88%) (Anderson *et al.*, 1964). The presence of α1,4-linked acid units to galactose residues has been indicated by Stephen & Schelpe (1964) and chromatographic evidence for the presence of the four aldobiouronic acids,

6-O-(β-D-glucopyranosyluronic acid)-D-galactose,

6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose,

4-O-(α-D-glucopyranosyluronic acid)-D-galactose,

4-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-galactose,

has been reported by Cree (1966). The results of a more detailed examination of *A.arabica* gum are reported and discussed in this chapter.

4.2) Results

Purification of A.arabica gum.- The gum (160 g.) was

dissolved in water (3 l.), filtered, dialysed, and electrodialysed. The polysaccharide was isolated as the freeze-dried product (152 g.), $[\alpha]_D$ +112° (c, 1.24) (Found: ash, 0.02%; N, 0.07%; OMe, 0.88%; equivalent weight, 1880; uronic acid, 10%; galactose, 32%; arabinose, 57%; rhamnose, 0.4%).

The gum was shown to migrate as a single band on electrophoresis on glass-fibre paper in 2N-sodium hydroxide solution (Lewis & Smith, 1957), on Whatman No.1 paper in 0.1M-ammonium carbonate buffer (pH, 8.9), and on cellulose acetate film in both 0.1M-ammonium carbonate buffer (pH, 8.9) and 0.1M-acetate buffer (pH, 4.7) (See Appendix II).

The gum was chromatographed on a DEAE-cellulose column (30 x 1.5 cm.) (Jermyn, 1962). Gradient elution with sodium chloride solution (0.0 → 0.3M) in 0.02M-acetate buffer (pH, 4.1) gave the elution pattern shown in Figure 1.

Separation and characterisation of neutral sugars.- The gum (1 g.) was hydrolysed with N-sulphuric acid (50 ml.) for 10 hours on a boiling-water bath, and the cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated to a syrup (921 mg.). The sugars were separated on Whatman No.3MM papers in solvent (a) to give three pure monosaccharides.

Fraction 1 (212 mg.) had $[\alpha]_D$ +80° (equil.) (c, 1.0) and had the same paper chromatographic mobility as D-galactose. The sugar, after recrystallisation from ethanol, had m.p. 162°; its X-ray diffraction pattern was identical to that of an authentic

specimen of D-galactose.

Fraction 2 (506 mg.) had $[\alpha]_D$ +104° (equil.) (c, 1.0) and had the same paper chromatographic mobility as L-arabinose. The sugar, after recrystallisation from aqueous ethanol, had m.p. 159°; its X-ray diffraction pattern was identical to that of an authentic specimen of L-arabinose.

Fraction 3 (2 mg.) was paper chromatographically identical to rhamnose in solvents (a), (b), (c), and (e).

Preparation of degraded gum A.- The gum (20 g.) was hydrolysed with 0.01N-sulphuric acid (800 ml.) for 100 hours on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and dialysed against water (2.5 l.). Dialysis was completed against running tap-water, and freeze-drying gave degraded gum A (4 g., yield, 20%), $[\alpha]_D$ +33° (c, 1.04) (Found: uronic acid, 11%; galactose, 86%; arabinose, 2%). [A sample (50 mg.) of degraded gum A was dissolved in water (10 ml.) and reduced with sodium borohydride (50 mg.). After dialysis, the freeze-dried product was hydrolysed; paper chromatographic examination of the hydrolysate in solvent (h) indicated the presence of galactitol; no arabinitol was detected]. The diffusate was concentrated to a syrup (11 g.), a portion (2 g.) of which was chromatographed on Whatman No.3MM papers to give two pure disaccharides.

Fraction 1 (89 mg.) had $[\alpha]_D$ +193° (c, 1.78) and yielded arabinose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 0.83 in solvent (a), 0.74 in

solvent (b), and 0.92 in solvent (e)] as 3-O-β-L-arabinopyranosyl-L-arabinose. A portion (21 mg.) of this disaccharide was methylated with methyl iodide and silver oxide in N,N-dimethylformamide. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4-tri- (i, T 1.02; ii, T 0.83), and 2,5- (i, T 1.79, 3.30; ii, T 1.25, 2.21) and 2,4-di-O-methyl-L-arabinose (i, T 2.07, 2.17; ii, T 1,42, 1.50). Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (c) showed the presence of 2,3,4-tri- (R_G 0.81), and 2,5- (R_G 0.85) and 2,4-di-O-methyl-L-arabinose (R_G 0.65). The phenylosazone of the disaccharide was prepared; after recrystallisation from ethanol, it had m.p. 229° - 232° (decomp.) (Lit. 233° - 235° (Bailey, 1965)].

Fraction 2 (28 mg.) was re-chromatographed on Whatman No. 3MM papers in solvent (f) to give a syrup (8.7 mg.), which had $[\alpha]_D$ +90° (c, 0.17) and yielded arabinose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 1.23 in solvent (b) and 1.41 in solvent (f)] as 3-O-β-L-arabinofuranosyl-L-arabinose. A portion (2 mg.) of the disaccharide was methylated with methyl iodide and silver oxide in N,N-dimethylformamide. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,5-tri- (i, T 0.58, 0.72; ii, T 0.51, 0.63), and 2,5- (i, T 1.78; 3.29; ii, T 1.27, 2.22) and 2,4-di-O-methyl-L-arabinose (i, T 2.07, 2.17; ii, T 1.41, 1.49).

Partial acid hydrolysis of degraded gum A.- Degraded gum A (2 g.) was hydrolysed with 0.5N-sulphuric acid (500 ml.) for 1 hour on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated to a syrup (1.9 g.). The syrup was chromatographed on Whatman No.3MM papers in solvent (a) to give two pure disaccharides.

Fraction 1 (39 mg.) had $[\alpha]_D$ +64° (c, 0.78) and yielded galactose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 0.47 in solvent (a) and 0.51 in solvent (e)] as an authentic sample of 3-O-β-D-galactopyranosyl-D-galactose. A portion (10 mg.) of the disaccharide was methylated with methyl iodide and silver oxide in N,N-dimethylformamide. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra- (i, T 1.67; ii, T 1.64), and 2,4,6- (i, T 3.72, 4.17; ii, T 2.98, 3.45) and 2,5,6-tri-O-methyl-D-galactose (i, T 3.99; ii, T 3.27). Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (c) showed the presence of 2,3,4,6-tetra- (R_G 0.90), and 2,4,6- (R_G 0.72) and 2,5,6-tri-O-methyl-D-galactose (R_G 0.88). The disaccharide was crystallised from aqueous acetone to give needles of 3-O-β-D-galactopyranosyl-D-galactose monohydrate, which had m.p. and mixed m.p. 156° - 159°.

Fraction 2 (55 mg.) had $[\alpha]_D$ +31° (c, 1.10) and yielded galactose on acid hydrolysis. It had the same paper

chromatographic mobility [R_{gal} 0.29 in solvent (a) and 0.35 in solvent (e)] as an authentic sample of 6-O-β-D-galactopyranosyl-D-galactose. A portion (10 mg.) of the disaccharide was methylated with methyl iodide and silver oxide in N,N-dimethylformamide. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra- (i, T 1.69; ii, T 1.67), and 2,3,5- (i, T 4.03, 5.37; ii, T 3.33, 4.37) and 2,3,4-tri-O-methyl-D-galactose (i, T 6.47; ii, T 5.29). Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (c) showed the presence of 2,3,4,6-tetra- (R_G 0.89), and 2,3,5- (R_G 0.87) and 2,3,4-tri-O-methyl-D-galactose (R_G 0.70).

Degraded gum A (100 mg.) was hydrolysed with N-sulphuric acid (20 ml.) for 7 hours on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated. Paper chromatography in solvent (b) indicated the presence of four aldobiouronic acids with the mobilities of 6-O-(β-D-glucopyranosyluronic acid)-D-galactose (R_{gal} 0.21), 4-O-(α-D-glucopyranosyluronic acid)-D-galactose (R_{gal} 0.28), 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose (R_{gal} 0.66), and 4-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-galactose (R_{gal} 0.70).

Methylation of degraded gum A.- Degraded gum A (1.050 g.) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give a product (926 mg.), $[\alpha]_D$ +14° (c, 1.18 in $CHCl_3$) (Found: OMe, 40.4%,

not raised on further attempted methylation). Methanolysis of a sample of this product followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 1; Figure 2 records a typical gas chromatogram obtained from column (ii). Examination of a hydrolysate of the methyl glycosides by paper chromatography in solvents (c) and (f) indicated the presence of 2-O-methylgalactose, in addition to those O-methyl sugars already characterised by g.l.c. of their methyl glycosides (Table 1).

Methylated degraded gum A (750 mg.) was heated under reflux for 8 hours with methanolic 5% hydrogen chloride (75 ml.). The resulting mixture of methyl glycosides was hydrolysed with N-sulphuric acid (100 ml.) on a boiling-water bath for 4 hours; the cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated to a syrup (700 mg.). The mixture of O-methyl sugars was separated on Whatman No.3MM papers by double development (Anderson & Rees, 1966) in solvent (f) to give eight fractions.

Fraction 1 (52 mg.) had $[\alpha]_D$ +116° (c, 1.04). It had the same paper chromatographic mobility [R_G 0.87 in solvent (c) and 0.72 in solvent (f)] as an authentic sample of 2,3,4,6-tetra-O-methyl-D-galactose. It was characterised by conversion into N-phenyl-2,3,4,6-tetra-O-methyl-D-galactosylamine, which, after recrystallisation from ethyl acetate, had m.p. 190° - 192° (Lit. m.p. 192° - 197° (Whistler, 1965)].

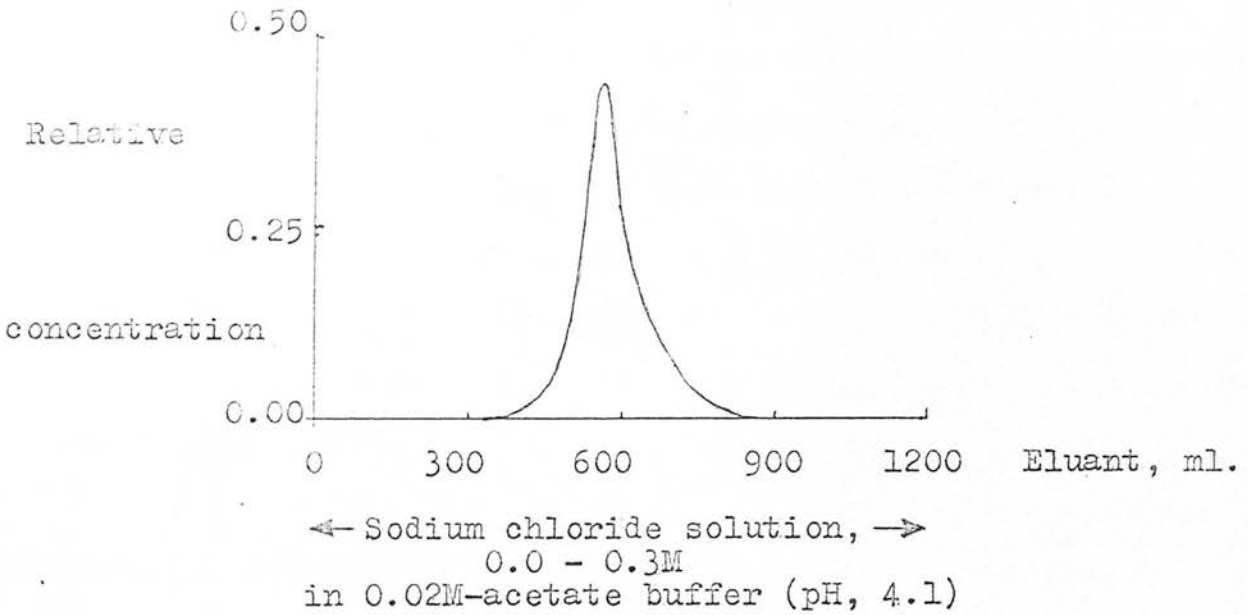


Figure 1. Elution pattern for a sample of A.arabica gum on a DEAE-cellulose column (30 x 1.5 cm.).

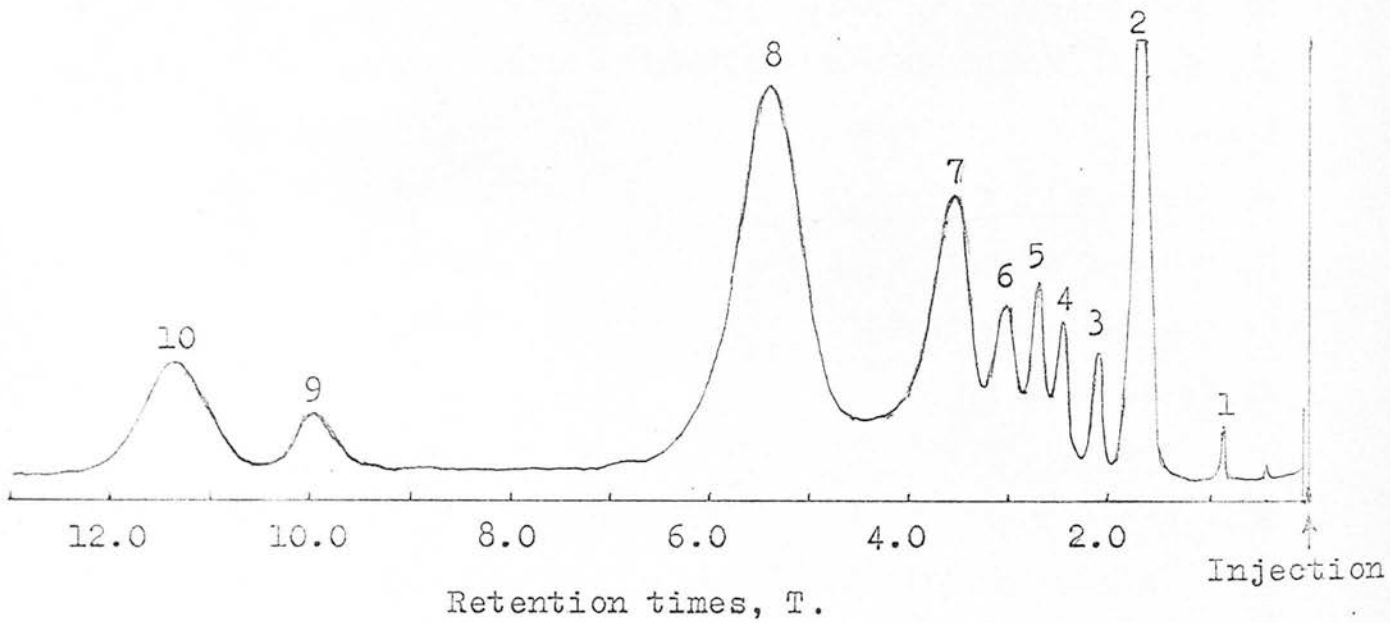


Figure 2. Gas chromatogram [column (ii)] of the methanolysis products from methylated degraded gum A.

Table 1

Examination of methanolysis and hydrolysis products from methylated degraded gum A.

O-Methyl sugars	Relative retention times, T, of methyl glycosides on columns:- (i)	(ii)	Peak numbers[a]	R_G in solvent (c)	Approx. relative molar props.
2,3,4-Me_3-arabinose	0.97	0.85	1	0.80	Trace
2,3,4,6-Me_4-galactose	1.67	1.66	2	0.87	++
2,3,6-Me_3-galactose	(2.88)[b]	2.30	4	0.72	+/2
	(3.70)	(3.04)	6		
	(4.12)	(3.46)	7		
2,4,6-Me_3-galactose	(3.70)	(3.04)	6	0.72	++
	(4.12)	(3.46)	7		
2,3,4-Me_3-galactose	6.38	5.27	8	0.72	+++
2,4-Me_2-galactose	14.4	9.8	9	0.50	+++
	16.3	11.4	10		
2,3,4-Me_3-glucuronic acid[c]	2.25	2.14	3	-	++
	2.88	2.74	5		
2-Me-galactose	-	-	-	0.33	+

[a] Peak numbers refer to those in Figure 2.

[b] Figures in parentheses indicate T values of components not completely resolved.

[c] As methyl ester methyl glycoside.

Fraction 2 (59 mg.) had $[\alpha]_D$ +102° (c, 1.18). Demethylation

gave galactose and arabinose. Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides indicated the presence of 2,3,4,6-tetra- (i, T 1.65; ii, T 1.67) and 2,3,6-tri-O-methyl-D-galactose (i, T 2.81, 3.79, 4.17; ii, T 2.36, 3.06, 3.47), and 2,3,4-tri-O-methyl-L-arabinose (i, T 0.97; ii, T 0.83).

Fraction 3 (48 mg.) had $[\alpha]_D$ +98° (c, 0.96). Methanolysis followed by g.l.c. examination of the methyl glycosides indicated the presence of a mixture of approximately equimolar proportions of 2,4,6- (i, T 3.73, 4.17; ii, T 3.04, 3.47) and 2,3,4-tri-O-methyl-D-galactose (i, T 6.41; ii, T 5.27). The mixture of O-methyl sugars was re-chromatographed in solvent (f) to obtain a syrup (12 mg.) of almost pure 2,4,6-tri-O-methyl-D-galactose. It was characterised by conversion into N-phenyl-2,4,6-tri-O-methyl-D-galactosylamine; after recrystallisation, twice from ethyl acetate and once from acetone:ether:light petroleum [1:1:1 (by vol.)], the needle shaped crystals gave m.p. 163° - 165° [Lit m.p. 170° - 180° (Whistler, 1965)].

Fraction 4 (54 mg.) had $[\alpha]_D$ +119° (c, 1.08). It had the same paper chromatographic mobility [R_G 0.70 in solvent (c)] as 2,3,4-tri-O-methyl-D-galactose and was characterised by conversion into N-phenyl-2,3,4-tri-O-methyl-D-galactosylamine; after recrystallisation from ethyl acetate, it had m.p. 163° - 165° [Lit. m.p. 164° - 169° (Whistler, 1965)]. Methanolysis of a portion (5 mg.) followed by g.l.c. examination of the methyl glycosides indicated the presence of 2,3,4-tri-O-methyl-D-galactose (i, T 6.38; ii, T 5.26).

Fraction 5 (44.5 mg.) had $[\alpha]_D$ +80° (c, 0.89). It had the same paper chromatographic mobility [R_G 0.50 in solvent (c) and 0.18 in solvent (f)] and paper electrophoretic mobility [M_G 0.26, 10 volts/cm., 0.5 ma./cm.] as 2,6-di-O-methyl-D-galactose. A portion (2 mg.) of the fraction was submitted to periodate oxidation; paper chromatographic examination of the oxidation products in solvent (c) revealed a brilliant yellow spot (p-anisidine hydrochloride spray) with the same mobility (R_G 0.20) as 2-O-methylmalondialdehyde.

Fraction 6 (69.5 mg.) had $[\alpha]_D$ +87° (c, 1.39). It had the same paper chromatographic mobility [R_G 0.46 in solvent (c) and 0.12 in solvent (f)] as an authentic sample of 2,4-di-O-methyl-D-galactose. Recrystallisation of the O-methyl sugar from acetone containing 1% of water gave 2,4-di-O-methyl-D-galactose monohydrate, m.p. 100° - 102° [Lit. m.p. 100° - 108° (Whistler, 1965)]; the crystals gave an X-ray powder photograph identical to that obtained for an authentic sample of 2,4-di-O-methyl D-galactose monohydrate. The derived N-phenyl-2,4-di-O-methyl-D-galactosylamine, after recrystallisation from acetone, had m.p. 214° - 216° [Lit. m.p. 214° - 228° (Whistler, 1965)].

Fraction 7 (20 mg.) had $[\alpha]_D$ +83° (c, 0.40). It had the same paper chromatographic mobility [R_G 0.29 in solvent (c) and 0.05 in solvent (f)] and paper electrophoretic mobility (M_G 0.41, 10 volts/cm., 0.5 ma./cm.] as an authentic sample of 2-O-methyl-D-galactose. Recrystallisation of the O-methyl sugar from glacial acetic acid gave crystals with m.p. 156° -

158° [Lit. m.p. 145° - 148° (Whistler, 1965)]; the crystals gave an X-ray powder photograph identical to that obtained from an authentic sample of 2-O-methyl-D-galactose.

Fraction 8 (43 mg.) had $[\alpha]_D$ +50° (c, 0.86). It had the same paper chromatographic mobility [R_G 0.88 in solvent (d)] as 2,3,4-tri-O-methyl-D-glucuronic acid. Methanolysis followed by g.l.c. examination of the methyl glycosides indicated the presence of the methyl ester of 2,3,4-tri-O-methyl-D-glucuronic acid (i, T 2.23, 2.80; ii, T 2.08, 2.66).

Reduction of methylated degraded gum A.- Lithium aluminium hydride (60 mg.) was added to methylated degraded gum A (60 mg.), dissolved in tetrahydrofuran (10 ml.); the mixture was refluxed for 3 hours. On cooling, excess of hydride was destroyed by addition of ethyl acetate and water; the mixture was reduced to dryness and exhaustively extracted with chloroform; the chloroform extract was concentrated to a syrup, which was methylated with methyl iodide (1 ml.) and silver oxide (1 g.) in N,N-dimethylformamide (1 ml.) to give a product (41 mg.), $[\alpha]_D$ +16° (c, 0.82 in $CHCl_3$). Methanolysis of this product followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 2. Examination of a hydrolysate of the methyl glycosides by paper chromatography in solvents (c) and (f) showed the presence of 2,3,4,6-tetra-O-methylglucose in addition to those neutral O-methyl sugars already identified in methylated degraded gum A.

Partial methanolysis of degraded gum A.- Degraded gum A

* Methylated product.

Table 2

Examination of methanolysis and hydrolysis products from methylated reduced degraded gum A.

O-Methyl sugars	Relative retention times, T, of methyl glycosides on columns:- (i)	(ii)	RG in solvent (f)	Approx. relative molar props.
2,3,4-Me_3-arabinose	(1.00)[a]	0.86	-	Trace
2,3,4,6-Me_4-glucose	(1.00)	1.00	1.00	++
	1.39	1.37		
2,3,4,6-Me_4-galactose	1.68	1.67	0.78	++
2,3,6-Me_3-galactose	2.88	2.31	0.49	+/2
	(3.70)	(3.00)		
	(4.12)	(3.45)		
2,4,6-Me_3-galactose	(3.70)	(3.00)	0.39	++
	(4.12)	(3.45)		
2,3,4-Me_3-galactose	6.35	5.25	0.33	+++
2,4-Me_2-galactose	14.3	9.9	0.11	+++
	16.2	11.3		
2-Me-galactose	-	-	0.05	+

[a] Figures in parentheses indicate T values of components not completely resolved.

(100 mg.) was refluxed with methanolic 2% hydrogen chloride (15 ml.) for 2 hours; the cooled methanolic solution was neutralised with silver carbonate, treated with hydrogen sulphide,

filtered, and concentrated to a syrup, which was hydrolysed with 0.5N-sulphuric acid (10 ml.) for 5 hours on a boiling-water bath; the cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and adsorbed on to a column (25 x 2.5 cm.) of Duolite A4 in the formate form. After elution of the neutral O-methyl sugars with water (3 l.), the acidic O-methyl sugars were eluted with aqueous 5% formic acid (500 ml.). Water and formic acid were removed on a rotary evaporator; paper chromatography in solvents (c) and (f) of the resulting syrup indicated that it was free of neutral O-methyl sugars. Methanolysis (methanolic 5% hydrogen chloride under reflux for 16 hours) of the syrup followed by g.l.c. examination of the products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4-tri-O-methyl-D-glucuronic acid (methyl ester, +++) [i, T 2.22, (2.82); ii, T 2.06, 2.65]. 2,3,6- (+)[i, T (2.82), 3.80, 4.17; ii, T 2.35, 3.07, 3.47] and 2,3,4-tri- (++) (i, T 6.40; ii, T 5.26), and 2,4-di-O-methyl-D-galactose (+/2) (i, T 14.2, 16.3; ii, T 9.9, 11.2). Examination of a hydrolysate of the methyl glycosides by paper chromatography in solvents (c), (d), and (f) confirmed the presence of these O-methyl sugars.

Smith degradation of degraded gum A.- Degraded gum A (1 g., dry wt.) was dissolved in water (25 ml.) and 0.05M-sodium metaperiodate solution (25 ml.) was added. The amount of periodate consumed after 96 hours was 6.35 mmoles/g. and the amount of formic acid released in this time was 2.9 mmoles/g.

After 96 hours, the reaction was stopped by addition of ethylene glycol (2 ml.). The solution was dialysed against running tap-water for 2 days. Sodium borohydride (500 mg.) was added and the mixture kept at room temperature for 30 hours. After dialysis for a further 2 days, the polyalcohol was hydrolysed in N-sulphuric acid at room temperature for 2 days; the solution was then neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and dialysed against water (500 ml.). The syrup obtained from the dialysate was shown by paper chromatography in solvents (b), (c), and (h) to contain glycerol and glycollic aldehyde as the main components, together with small amounts of some slower moving non-reducing glycosides. After further dialysis against running tap-water for 2 days, degraded gum B (85 mg., dry wt., yield 8.5%), $[\alpha]_D$ +20° (c, 0.85) was isolated as the freeze-dried product.

Partial acid hydrolysis and methylation of degraded gum B.- Degraded gum B (10 mg.) was hydrolysed with 0.5N-sulphuric acid (5 ml.) for 1 hour on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated. Paper chromatographic examination of the syrup in solvent (a) indicated the presence of galactose, two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose (R_{gal} 0.47) and 6-O-β-D-galactopyranosyl-D-galactose (R_{gal} 0.29), and higher neutral oligosaccharides.

Degraded gum B (50 mg.) was methylated successively with

dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide in N,N-dimethylformamide. Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 3; Figure 3 records a typical gas chromatogram obtained from column (ii). Examination of a hydrolysate of the methyl glycosides by paper chromatography in solvents (c) and (f) indicated the presence of 2,6-di- and 2-O-methylgalactose, in addition to those O-methyl sugars already characterised by g.l.c. of their methyl glycosides.

Methylation of A.arabica gum.- The purified gum (10 g.) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide (the quantities of reagents used in the procedures outlined in Chapter II on pages 11 and 12 were scaled up ten times), to give a product (5.6 g.), $[\alpha]_D$ +77° (c, 0.93 in $CHCl_3$) (Found: OMe, 38.2%, not raised on further attempted methylation). Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 4; Figure 4 records a typical gas chromatogram obtained from column (ii).

The methylated gum (5 g.) was suspended in 2N-sulphuric acid (75 ml.) and kept at room temperature until dissolution was almost complete (4 days). Water (75 ml.) was added and the solution warmed for 2 hours at 50°C before being heated on a boiling-water bath for 12 hours. The cooled solution was neutralised with barium carbonate, filtered, and treated with Amberlite resin IR-120 (H). Separation of neutral O-methyl sugars

Table 3

Examination of methanolysis products from methylated degraded gum B

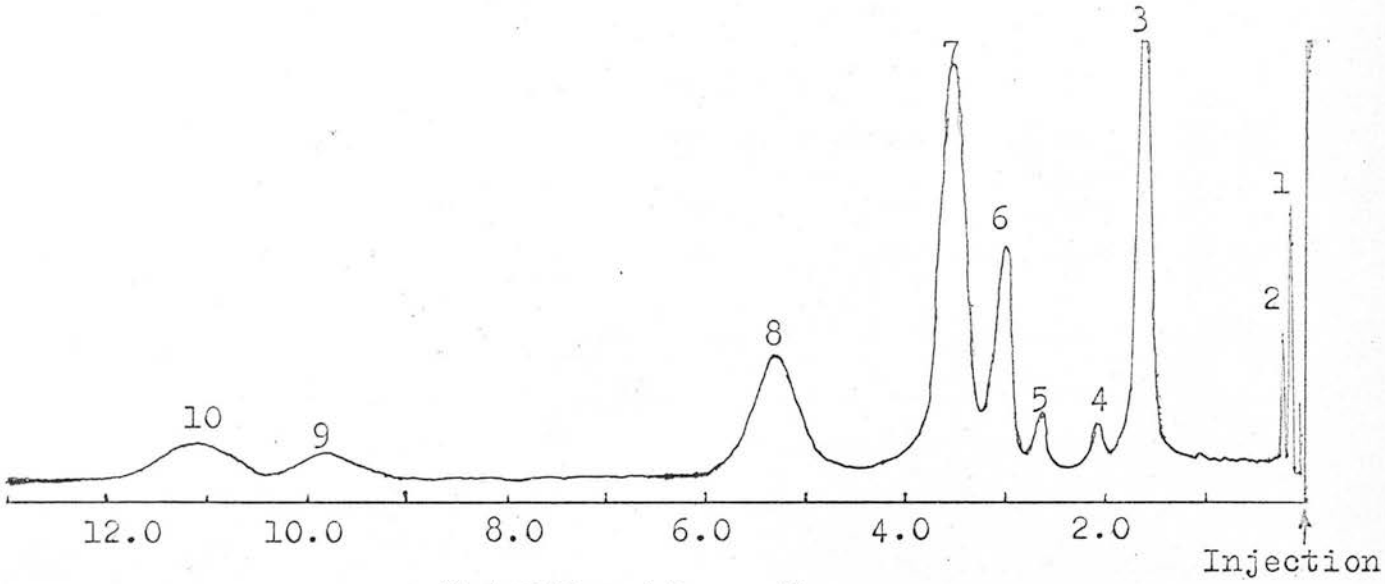
O-Methyl sugars	Relative retention times, T, of methyl glycosides on columns:- (i)	(ii)	Peak[a] numbers	Approx. relative molar props.
2,3,4,6-Me_4-galactose	1.68	1.67	3	++
2,4,6-Me_3-galactose	3.72	2.99	6	++++
	4.16	3.45	7	
2,3,4-Me_3-galactose	6.40	5.26	8	+
2,4-Me_2-galactose	14.6	9.8	9	++
	16.1	11.2	10	
2,3,4-Me_3-glucuronic acid[b]	2.32	2.08	4	Trace
	2.96	2.67	5	
Unknown peaks	0.21	0.20	1	
	0.32	0.30	2	

[a] Peak numbers refer to those in Figure 3.

[b] As methyl ester methyl glycoside.

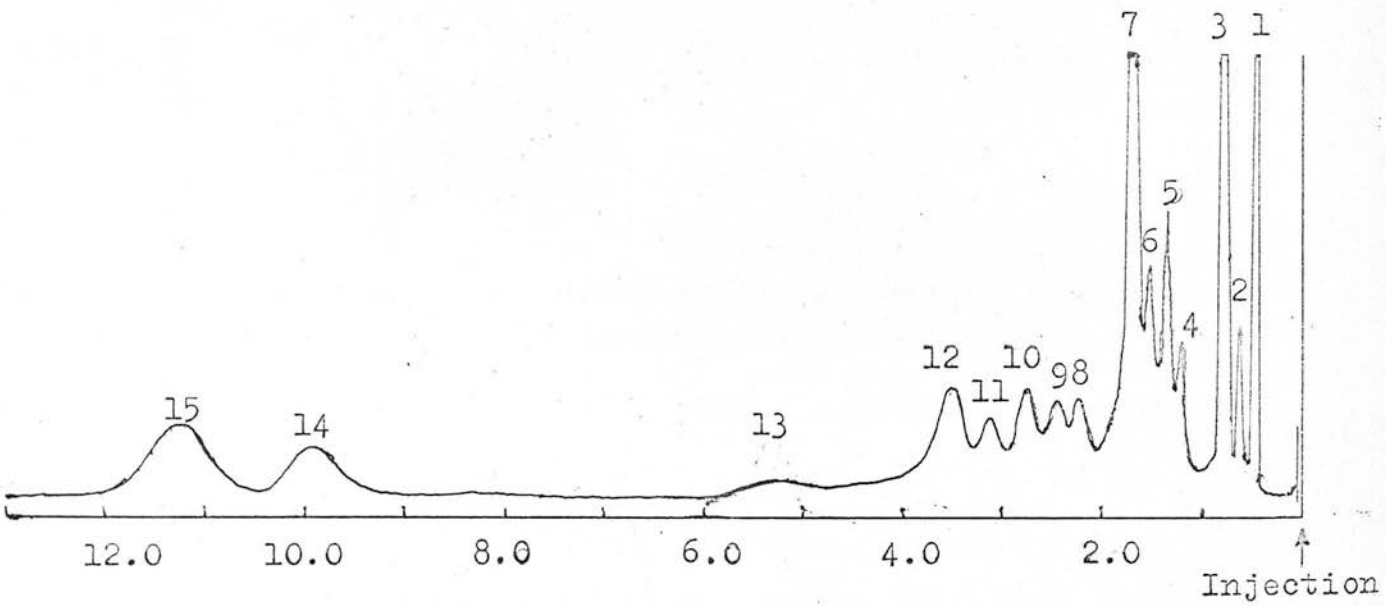
from acidic O-methyl sugars was accomplished by ion-exchange chromatography on a column (30 x 2.5 cm.) of Duolite A4 in the formate form. Elution of the column with water (2 l.) gave the neutral O-methyl sugar fraction; elution with 5% formic acid (500 ml.) gave the acidic O-methyl sugar fraction.

Concentration of the acidic O-methyl sugar fraction yielded



Retention times, T.

Figure 3. Gas chromatogram [column (ii)] of the methanolysis products from methylated degraded gum B.



Retention times, T.

Figure 4. Gas chromatogram [column (ii)] of the methanolysis products from methylated A.arabica gum.

Table 4

Examination of methanolysis products from methylated A.arabica gum

O-Methyl sugars	Relative retention times, T, of methyl glycosides on columns:- (i)	(ii)	Peak[a] numbers
2,3,4-Me_3-rhamnose	0.49	(0.51)[b]	1
2,3,5-Me_3-arabinose	0.57	(0.51)	1
	0.74	0.67	2
2,3,4-Me_3-arabinose	(1.04)	(0.85)	3
2,5-Me_2-arabinose	1.80	1.24	4
		(2.21)	8
3,5-Me_2-arabinose	(1.04)	(0.85)	3
	2.35	1.76	7
3,4-Me_2-arabinose	1.95	1.37	5
2,3,4,6-Me_4-galactose	1.67	1.66	6
2,3,6-Me_3-galactose		2.31	9
		(3.03)	11
		(3.45)	12
2,4,6-Me_3-galactose	3.71	(3.03)	11
	4.13	(3.45)	12
2,3,4-Me_3-galactose	6.39	5.24	13
2,4-Me_2-galactose	14.4	9.9	14
	16.3	11.3	15
2,3,4-Me_3-glucuronic acid[c]	2.21	(2.21)	8
	2.94	2.80	10

[a] Peak numbers refer to those in Figure 4.

[b] Figures in parentheses indicate T values of components not completely resolved.

[c] As methyl ester methyl glycoside.

a syrup (180 mg.), which was refluxed with methanolic 2% hydrogen chloride overnight. After neutralisation with silver carbonate, filtration, and concentration to a syrup, the methyl ester methyl glycosides were reduced by refluxing in dry tetrahydrofuran (40 ml.) with lithium aluminium hydride (300 mg.) for 2 hours. Excess of lithium aluminium hydride was destroyed by careful addition of water to the cooled reaction mixture; tetrahydrofuran and water were removed on a rotary evaporator, and the residue extracted exhaustively with chloroform and acetone. Concentration of the extract gave a syrup, which was hydrolysed with 0.5N-sulphuric acid (10 ml.) for 4 hours on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated to a syrup (80 mg.). Paper chromatographic examination of this product indicated the presence of 2,3,4-tri-O-methyl-D-glucose ([R_G 0.88 in solvent (c) and 0.69 in solvent (f)] as the main component. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4-tri-O-methyl-D-glucose (i, T 2.43, 3.38).

Concentration of the neutral O-methyl sugars yielded a syrup (3.9 g.). A portion (3.5 g.) of this syrup was

chromatographed on a cellulose column (100 x 3.5 cm.) with light petroleum (b.p. 100° - 120°)-butan-1-ol (7:3, satd. with water) as eluant, to give five fractions, and with light petroleum (b.p. 100° - 120°)-butan-1-ol (1:1, satd. with water) as eluant, to give a further five fractions.

Fraction 1 (360 mg.) from tubes 1-180 had $[\alpha]_D$ -18° (c, 2.14). It had the same paper chromatographic mobility [R_G 0.99 in solvent (c) and 1.0 in solvent (f)] as 2,3,5-tri-O-methyl-L-arabinose. It was characterised by conversion into 2,3,5-tri-O-methyl-L-arabonamide; after recrystallisation from acetone, the needle shaped crystals gave m.p. 135° - 136° [Lit. m.p. 134° - 138° (Whistler, 1965)].

Fraction 2 (65 mg.) from tubes 181-260 had $[\alpha]_D$ +31° (c, 1.30). Demethylation gave galactose and arabinose. Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides indicated the presence of 2,3,4,6-tetra-O-methyl-D-galactose (i, T 1.67) and 2,3,5-tri- (i, T 0.58, 0.72), and 2,5- (i, T 1.75, 3.25) and 3,5-di-O-methyl-L-arabinose (i, T 1.05, 2.32).

Fraction 3 (685 mg.) from tubes 261-400 had $[\alpha]_D$ +25° (6, 1.06). Methanolysis followed by g.l.c. examination of the methyl glycosides indicated that 2,5- (i, T 1.78, 3.27) and 3,5-di-O-methyl-L-arabinose (i, T 1.07, 2.33) were the main components of the mixture. A portion (500 mg.) of the syrup was chromatographed on Whatman No.3MM papers in solvent (c) to obtain a syrupy mixture (313 mg.) of 2,5- and 3,5-di-O-methyl-

L-arabinose. Paper electrophoretic examination confirmed the presence of 2,5- (M_G 0.00, 10 volts/cm., 0.5 ma./cm.) and 3,5-di-O-methyl-L-arabinose [M_G 0.70, 10 volts/cm., 0.5 ma./cm., brown spot (yellow under ultraviolet) with aniline oxalate]. These components were separated from the syrupy mixture (150 mg.) by continuous electrophoresis at 600 volts on a sheet of Whatman No.54 paper in 0.05M-borate buffer (pH, 9.2) for 24 hours. On drying, the electrophoretogram was sprayed with p-anisidine hydrochloride and developed. Migration towards the anode of 3,5-di-O-methyl-L-arabinose as its borate complex permitted separation of this O-methyl sugar from its 2,5-di-O-methyl isomer into tubes at the bottom of the paper curtain. Tubes containing 2,5-di-O-methyl-L-arabinose were combined, borate was removed as methyl borate by repeated evaporation with methanol, and the sugar (35 mg.) characterised by conversion into 2,5-di-O-methyl-L-arabonamide; after recrystallisation from ethyl acetate, it had m.p. 124° - 126° [Lit. m.p. 122° - 132° (Whistler, 1965)]. Tubes containing 3,5-di-O-methyl-L-arabinose were combined, borate was removed, and the sugar (27 mg.) characterised by conversion into 3,5-di-O-methyl-L-arabonamide; after recrystallisation from ethyl acetate, it had m.p. 143° - 144° [Lit. m.p. 144° - 145° (Whistler, 1965)].

Fraction 4 (346 mg.) from tubes 401-600 had $[\alpha]_D$ +95° (c, 1.23). Paper chromatographic examination indicated the presence of 2,3,4-tri-O-methyl-L-arabinose [R_G 0.78 in solvent (c) and 0.55 in solvent (f)] in addition to 2,5- and 3,5-di-O-methyl-

L-arabinose.

Fraction 5 (116 mg.) from tubes 601-1100 had $[\alpha]_D$ +93° (c, 1.12). Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides indicated that 2,4,6- [i, T (3.70), (4.10)], 2,3,6- [i, T 2.88, (3.70), (4.10)] and 2,3,4-tri-O-methyl-D-galactose (i, T 6.30) were the main components.

Fraction 6 (93 mg.) from tubes 1101-1400 had $[\alpha]_D$ +100° (c, 1.86). Methanolysis of a small portion (4 mg.) followed by g.l.c. examination of the methyl glycosides showed the presence of 3,4-di-O-methyl-L-arabinose (i, T 1.91). The remainder of the syrup was chromatographed on Whatman No.3MM papers in solvent (c) to give a product (23 mg.). Demethylation gave arabinose. On paper chromatographic and paper electrophoretic examination, it had R_G values of 0.55 in solvent (c) and 0.20 in solvent (f) and a M_G value of 0.21 (10 volts/cm., 0.5 ma./cm.). A portion (5 mg.) was converted into the methyl glycosides and methylated with methyl iodide (0.5 ml.) and silver oxide (500 mg.) in N,N-dimethylformamide (0.5 ml.). G.l.c. examination of the reaction mixture showed the presence of the methyl glycoside(s) of 2,3,4-tri-O-methyl-L-arabinose (i, T 0.97).

Fraction 7 (146 mg.) from tubes 1401-1500 had $[\alpha]_D$ +90° (c, 0.80). Paper chromatographic examination in solvents (c) and (f) showed the presence of 2,4-di-O-methyl-D-galactose as the main component of the mixture.

Fraction 8 (56 mg.) from tubes 1501-1640 had $[\alpha]_D$ +111°

(c, 1.12). This fraction was chromatographed on Whatman No.3MM papers in solvent (c) to give a product (19 mg.). Demethylation gave arabinose. The O-methyl sugar had the same chromatographic mobility [R_G 0.35 in solvent (c) and 0.08 in solvent (f)] as an authentic specimen of 4-O-methyl-L-arabinose kindly supplied by Dr. C. T. Bishop. It also had the same paper electrophoretic mobility [M_G 0.29, 10 volts/cm., 0.4 ma./cm.] as the authentic sample. A portion (5 mg.) was converted to the methyl glycosides and methylated (cf. Fraction 6). G.l.c. examination of the reaction mixture indicated the presence of the methyl glycoside(s) of 2,3,4-tri-O-methyl-L-arabinose (i, T 0.96).

Fraction 9 (66 mg.) from tubes 1641-1800 had $[\alpha]_D$ +91° (c, 1.32). Paper chromatographic examination in solvents (c) and (f) indicated the presence of 2-O-methyl-D-galactose as the main component of the mixture.

Fraction 10 (31 mg.) was collected from tubes 1801-2000. Paper chromatography in solvents (a) and (b) showed the presence of galactose and arabinose.

Preparation, partial acid hydrolysis, and methylation of polysaccharide I.- Whole gum (40.0 g., dry wt.) was dissolved in water (1 l.) and 0.25M-sodium metaperiodate solution (1 l.) was added. Results for the release of formic acid with time on periodate oxidation are shown in Table 5; the amount of periodate consumed by the whole gum in 96 hours was 3.01 mmoles/g. After 96 hours, the reaction was stopped by addition of ethylene glycol (20 ml.); the solution was dialysed against running tap-

Table 5

Formic acid released (mmoles/g.) on periodate oxidation

Time (hours)	1	3	6	12	24	48	72	96
A.arabica gum	0.14	0.20	0.25	0.32	0.36	0.49	0.60	0.69
Polysaccharide I	0.37	0.40	0.40	0.42	0.43	-	-	-
Polysaccharide II	0.79	0.80	0.84	0.91	0.95	-	-	-

water for 2 days. Sodium borohydride (10 g.) was added and the reaction mixture kept at room temperature for 30 hours. After dialysis for a further 2 days, the polyalcohol was hydrolysed in N-sulphuric acid at room temperature for 2 days; after dialysis of the acidic solution against running tap-water for 2 days, polysaccharide I(23.4 g., dry wt., yield, 59%), $[\alpha]_D$ +63° (c, 1.04) (Found: galactose, 41%; arabinose, 59%) was isolated as the freeze-dried product.

Polysaccharide I (20 mg.) was hydrolysed with 0.5N-sulphuric acid (6 ml.) for 1 hour on a boiling-water bath; the cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated. Paper chromatographic examination of the syrup indicated the presence of galactose, arabinose, two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose [R_{gal} 0.49 in solvent (a), 0.29 in solvent (b), and 0.53 in solvent (e), minor component] and 6-O-β-D-galactopyranosyl-D-galactose [R_{gal} 0.30 in solvent (a), 0.20 in solvent (b), and 0.38 in

Table 6

O-Methyl sugars present in methylated polysaccharides I-IV

O-Methyl sugar	I	II	III	IV
2,3,5-Me_3-arabinose	++	++	+	+
2,5-Me_2-arabinose	++	+	+	+
3,5-Me_2-arabinose	+++	++	+	+
2,3,4,6-Me_4-galactose	Trace	Trace	+	+
2,4,6-Me_3-galactose	+	+	++	++
2,3,4-Me_3-galactose	Trace	+	+	+
2,4-Me_2-galactose	++	++	+	+

solvent (e), major component], and higher neutral oligosaccharides.

Polysaccharide I (308 mg.) was methylated with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give a product (222 mg.), $[\alpha]_D$ +43° (c, 1.48 in $CHCl_3$) (Found: OMe, 39.9%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 6. Acid hydrolysis of the methyl glycosides, followed by paper chromatographic examination of the free sugars, confirmed these results.

Preparation, partial acid hydrolysis, and methylation of polysaccharide II.- Borohydride reduction of periodate-oxidised polysaccharide I (22.5 g., dry wt.), followed by controlled acid

hydrolysis at room temperature for 2 days, gave polysaccharide II (9.8 g., dry wt., yield, 44%), $[\alpha]_D$ +51° (c, 0.86) (Found: galactose 50%; arabinose 50%). Results for releases of formic acid with time on periodate oxidation of polysaccharide I are shown in Table 5; the amount of periodate consumed by polysaccharide I in 24 hours was 2.44 mmoles/g.

Partial acid hydrolysis of polysaccharide II (20 mg.), followed by paper chromatographic examination of the hydrolysate indicated the presence of galactose, arabinose, two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose [R_{gal} 0.45 in solvent (a) and 0.53 in solvent (e), minor component] and 6-O-β-D-galactopyranosyl-D-galactose [R_{gal} 0.26 in solvent (a) and 0.40 in solvent (e), major component], and higher neutral oligosaccharides.

Polysaccharide II (258 mg.) was methylated, as described for polysaccharide I, to give a product (152 mg.), $[\alpha]_D$ +30° (c, 1.14 in $CHCl_3$) (Found: OMe, 42.1%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 6. Acid hydrolysis of the methyl glycosides, followed by paper chromatographic examination of the free sugars, confirmed these results.

Preparation, partial acid hydrolysis, and methylation of polysaccharide III.- Borohydride reduction of periodate-oxidised polysaccharide II (8.50 g., dry wt.), followed by controlled

acid hydrolysis at room temperature for 2 days gave polysaccharide III (1.40 g., dry wt., yield, 17%), $[\alpha]_D$ +46° (c, 1.06) (Found: galactose 72%; arabinose 28%). Results for release of formic acid with time on periodate oxidation of polysaccharide II are shown in Table 5; the amount of periodate consumed by polysaccharide II in 24 hours was 3.28 mmoles/g.

Partial acid hydrolysis of polysaccharide III (20 mg.), followed by paper chromatographic examination of the hydrolysate indicate the presence of galactose, arabinose, two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose [R_{gal} 0.56 in solvent (e), minor component] and 6-O-β-D-galactopyranosyl-D-galactose [R_{gal} 0.43 in solvent (e), major component], and higher neutral oligosaccharides.

Polysaccharide III (110 mg.) was methylated, as described for polysaccharide I, to give a product (76 mg.), $[\alpha]_D$ +26° (c, 0.90 in $CHCl_3$) (Found: OMe, 42.9%; not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 6. Acid hydrolysis of the methyl glycosides, followed by paper chromatographic examination of the free sugars, confirmed these results.

Preparation and methylation of polysaccharide IV.- Borohydride reduction of periodate-oxidised polysaccharide III (1.00 g., dry wt.), followed by controlled acid hydrolysis at room temperature for 2 days, gave polysaccharide IV (25 mg., yield 2.5%). Acid hydrolysis yielded galactose and arabinose.

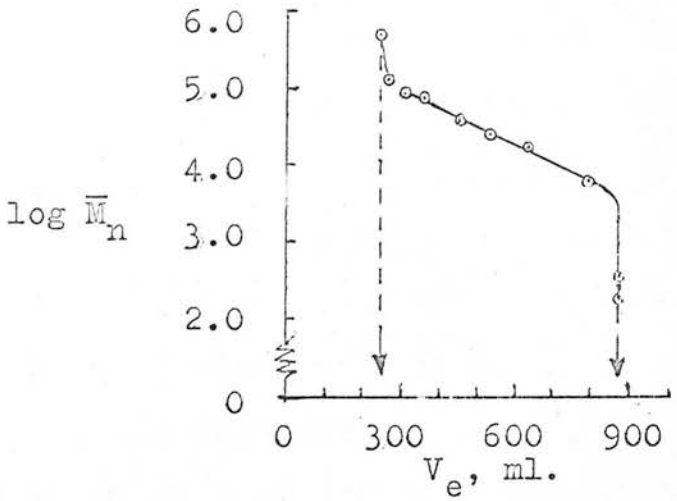


Figure 5. Plot of elution volume, V_e, against log $\overline{M}_n$ for dextran fractions of known $\overline{M}_n$ values. "Bio-Gel P300" column (50 x 4.8 cm.): elution with M-sodium chloride. The arrows correspond with those on Figure 6.

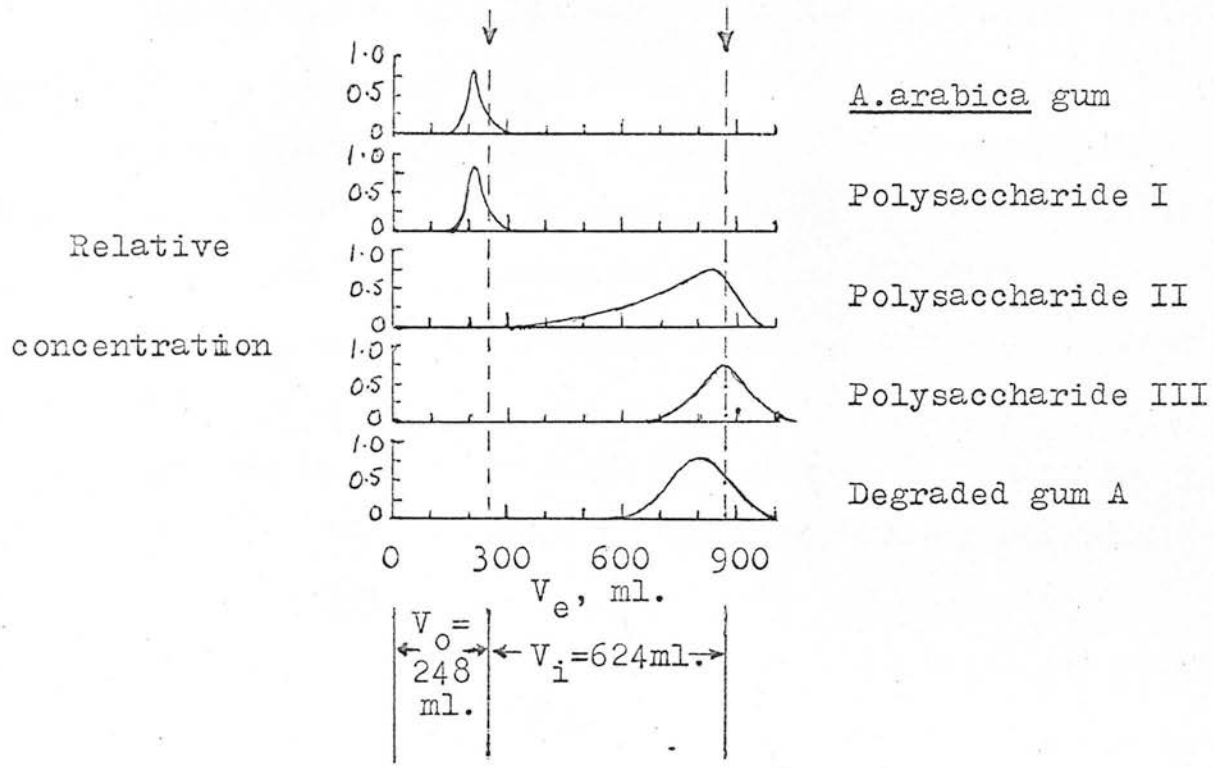


Figure 6. Elution patterns for A.arabica gum, polysaccharides I, II, and III, and degraded gum A. "Bio-Gel P300" column (50 x 4.8 cm.): elution with M-sodium chloride.

The amount of formic acid released on periodate oxidation of polysaccharide III in 48 hours was 0.99 mmoles/g.; the amount of periodate consumed was 3.58 mmoles/g.

Polysaccharide IV (20 mg.) was dissolved in N,N-dimethylformamide (5 ml.); methylation was carried out by shaking with methyl iodide (5 ml.) and silver oxide (2 g.). Methanolysis of the methylated product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 6.

Molecular-sieve chromatography.- Figure 5 shows a calibration plot of V_e against log $\bar{M}_n$ obtained with dextran fractions of known $\bar{M}_n$. Figure 6 shows the elution patterns obtained for A.arabica gum, polysaccharides I, II, and III, and degraded gum A. The elution volumes for both A.arabica gum and polysaccharide I preceed the "exclusion volume" of the column as indicated by the elution volume for "blue dextran." This somewhat surprising observation was reproducible on a second "Bio-Gel P300" column. It would therefore appear that the preparation of "blue dextran" supplied by Pharmacia Ltd., Uppsala, Sweden is not of sufficiently high molecular-weight to give the true exclusion volume of a "Bio-Gel P300" column. The elution volumes for A.arabica gum and polysaccharide I are indicative of very high number-average molecular-weights - perhaps greater than 500,000. The elution pattern for polysaccharide II indicates the presence of a high proportion of low molecular-weight material; an estimate of the $\bar{M}_n$ is made difficult by the asymmetric nature of the elution curve. The elution volume for polysaccharide III

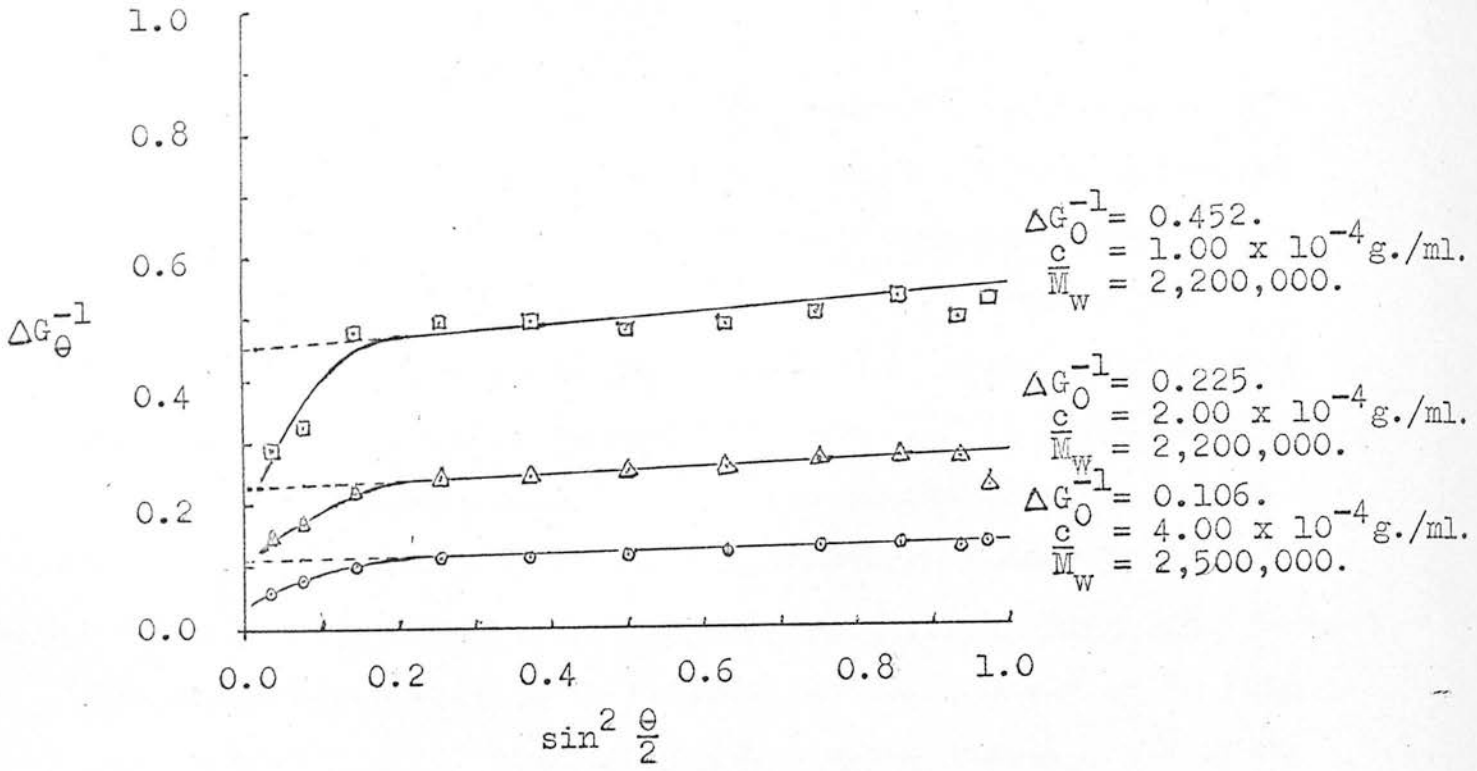


Figure 7. Plots of ΔG_θ^{-1} against $\sin^2 \frac{\theta}{2}$ for A.arabica gum.

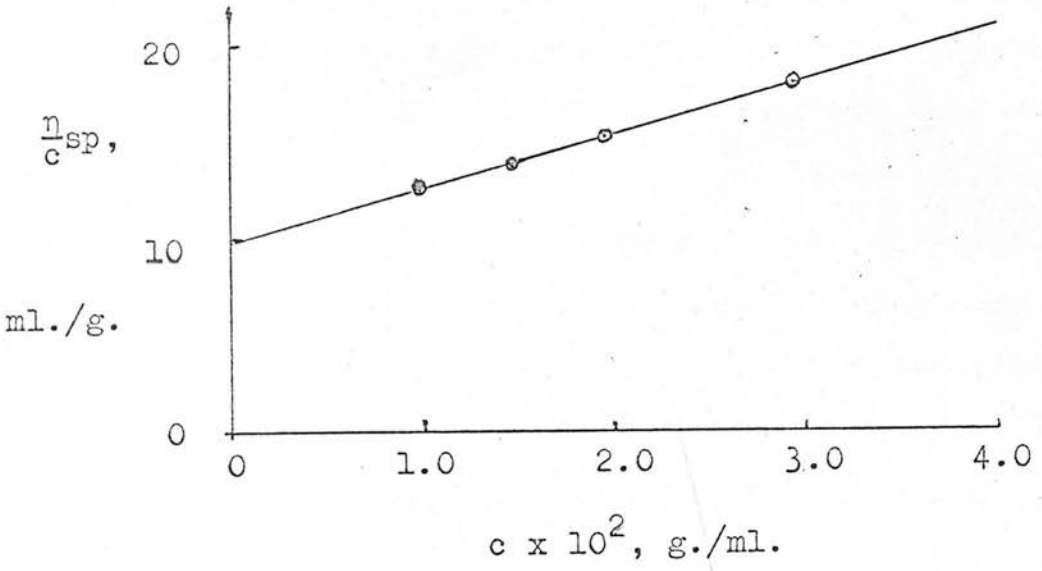


Figure 8. Plot of η_{sp}/c against c for A.arabica gum.

suggests a value for the $\bar{M}_n$ of less than 2,000. An estimated value of 5,400 was obtained for the $\bar{M}_n$ of degraded gum A.

Light-scattering and viscosity measurements.- Plots of ΔG_θ^{-1} against $\sin^2 \frac{\theta}{2}$ for A.arabica gum are shown in Figure 7. An average value of 2.3×10^6 was calculated for the $\bar{M}_w$.

A plot of η_{sp}/c against c for A.arabica gum is shown in Figure 8. A value of 9.9 ml./g. was obtained for the limiting viscosity number.

4.3) Discussion.

In preliminary experiments, samples of A.arabica gum were examined by zone electrophoresis (Appendix II) and ion-exchange chromatography on DEAE-cellulose (Jermyn, 1962). Since no sharp discontinuities in the properties of the molecular species were indicated by these experiments, it seems probable that A.arabica gum exhibits the same kind of heterogeneity as A.senegal gum (cf. Chapter III). If this is the case, then A.arabica gum may be considered to contain a continuous spectrum of related molecular species.

The gum is composed of residues of D-galactose (32%), L-arabinose (57%), L-rhamnose (0.4%), D-glucuronic acid (4%), and 4-O-methyl-D-glucuronic acid (6%). Although it proved impracticable to isolate sufficient quantities of rhamnose to enable a value for its specific rotation to be determined, the sugar was assumed to be present in the L-form because of its occurrence in this series in other Acacia gums (Smith &

Montgomery, 1959). The low rhamnose content of A.arabica gum and the high positive value of +112° for its specific rotation are not now considered to be atypical features within the Acacia group of plant gums (Anderson & Karamalla, 1966a).

Despite the fact that solutions of A.arabica gum have a lower limiting viscosity number ([η] = 9.9 ml./g.) than A.senegal gum solutions ([η] = 20.0 ml./g.), the gum from A.arabica has a higher weight-average molecular-weight ($\bar{M}_w$ = 2.3 x 10^6) than A.senegal gum ($\bar{M}_w$ = 6.0 x 10^5). Molecular-sieve chromatography had indicated a very high molecular-weight for A.arabica gum in advance of any light-scattering measurements. A comparison of the physico-chemical properties of A.arabica gum with those of A.senegal gum suggests that there are important differences in their molecular architecture in addition to fine structural variations, which are predictable from a knowledge of the differences in chemical composition of the two gums.

A few experiments of a physico-chemical nature directed towards some assessment of heterogeneity, and some appraisal of molecular properties such as size and shape, should always preceed any detailed structural investigations of a plant gum. Too often, such problems are either ignored or postponed until after structural investigations have been completed (cf. Banks & Greenwood, 1963).

Hydrolysis of the gum under controlled conditions with 0.01N-sulphuric acid resulted in the release of almost all the arabinose together with small amounts of galactose. Degraded

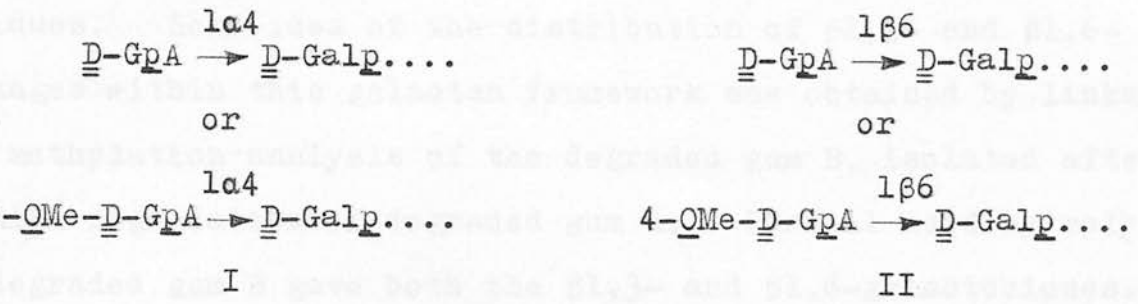
gum A, isolated after dialysis, contained galactose (86%), arabinose (2%), and uronic acid (11%). The value of $+33^{\circ}$ for the specific rotation of degraded gum A is somewhat different from the value of -11° obtained for autohydrolysed A.senegal gum (Chapter III). This difference may be partly explained by the configurations of the linkages involving uronic acid residues to galactose units. Degraded gum A contains some α1,4-linked uronic acid residues to galactose units, which are absent in autohydrolysed A.senegal gum. On the other hand, autohydrolysed A.senegal gum contains a higher proportion of β1,6-linked uronic acid residues to galactose units than degraded gum A. Differences in the arrangement of galactose residues within the galactan frameworks of the two degraded gums may also contribute to the difference in specific rotation. Molecular-sieve chromatography gave an estimated value of 5,400 for the $\bar{M}_n$ of degraded gum A. If the whole gum is based on a galactan framework then obviously considerable degradation of this framework has occurred during the mild conditions of controlled acid hydrolysis. This degradation does not appear to have been caused by the presence of any internal acid-labile arabinofuranose residues, since acid hydrolysis of borohydride-reduced degraded gum A yielded only galactitol and no arabinitol (Anderson & Stoddart, 1966c). There is no evidence for the presence of any galactofuranose residues in the gum; this implies that, just as in A.senegal gum (Chapter III), certain galactopyranosidic bonds must be unusually sensitive towards mild conditions of acid

hydrolysis. Smidsrød, Haug & Larsen (1966) have drawn attention to the fact that, when the overall proton concentration is low, the negative charge associated with acidic polysaccharides will cause the proton concentration in the region of the macromolecules to be higher than that in the bulk of the solution. These authors suggest that this effect may explain the unexpectedly high rates of acid hydrolysis of acidic polysaccharides above pH values of 1.

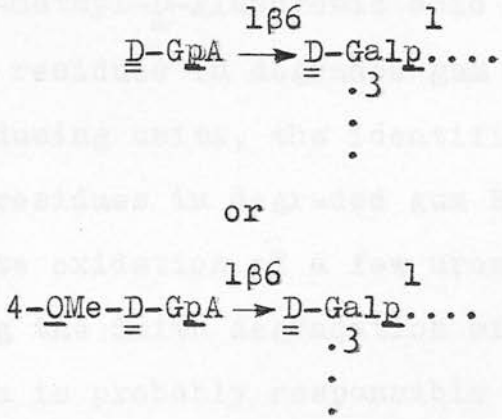
Partial acid hydrolysis of degraded gum A gave two galactobioses in addition to the four aldobiouronic acids already mentioned in the introduction. The first galactobiose had the same paper chromatographic mobility as 3-O-β-D-galactopyranosyl-D-galactose and was characterised as its crystalline monohydrate. Methylation analysis confirmed that a galactose unit was 3-O-substituted by a galactopyranose residue in the disaccharide. Methylation evidence for the second galactobiose indicated that a galactose unit was 6-O-substituted by a galactopyranose residue. The paper chromatographic behaviour of the disaccharide confirmed that it was identical to 6-O-β-D-galactopyranosyl-D-galactose. Both galactobioses have been isolated and characterised from A.senegal gum (Chapter III).

An examination of methylated degraded gum A showed the presence of 2,3,4-tri-O-methyl-L-arabinose (a trace), 2,3,4,6-tetra- (++), 2,3,6- (+/2), 2,4,6- (++), and 2,3,4-tri- (+++), 2,4-di- (+++), and 2-O-methyl-D-galactose (+), and 2,3,4-tri-O-methyl-D-glucuronic acid (++). These O-methyl sugars indicate

the presence of terminal non-reducing L-arabinopyranose, D-galactopyranose, and D-glucopyranosyluronic acid residues, and 4-O-, 3-O-, 6-O-, and 3,6-di-O-substituted D-galactopyranose units. The presence of some 2-O-methyl-D-galactose is ascribed to undermethylation. An examination of the methylated reduced product, obtained after reduction of methylated degraded gum A with lithium aluminium hydride (Abdel-Akher & Smith, 1950) followed by methylation, showed the presence of 2,3,4,6-tetra-O-methyl-D-glucose in addition to the neutral O-methyl sugars already mentioned. This confirms that D-glucuronic acid residues are present as non-reducing end-groups in degraded gum A. Since α1,4-linked aldobiouronic acids involving galactose residues have been identified in degraded gum A, the presence of some 4-O-substituted galactose units is to be expected. Likewise, β1,6-linked aldobiouronic acids account for the presence of some of the 6-O- and 3,6-di-O-substituted galactose units. Partial methanolysis of methylated degraded gum A has indicated the presence of the methylated aldobiouronic acids, 4-O-(2,3,4-tri-O-methyl-α-D-glucopyranosyluronic acid)-2,3,6-tri-O-methyl-D-galactose, 6-O-(2,3,4-tri-O-methyl-β-D-glucopyranosyluronic acid)-2,3,4-tri-O-methyl-D-galactose together with small amounts of 6-O-(2,3,4-tri-O-methyl-β-D-glucopyranosyluronic acid)-2,4-di-O-methyl-D-galactose. It follows that I, II, and III are possible structural fragments for residues attached to the galactan framework of degraded gum A. In addition to D-glucopyranosyluronic acid residues in such fragments as I, II, and III,



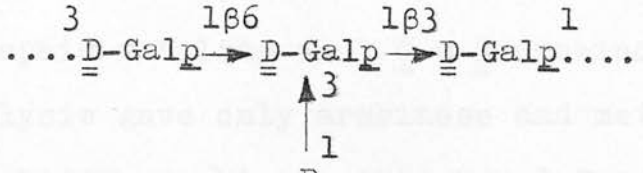
L-arabinopyranose (IV) and D-galactopyranose residues (V) occupy terminal non-reducing positions. The occurrence of a small



proportion of L-arabinopyranose residues attached directly to the galactan framework constitutes a feature that has not been exhibited by any other Acacia gum studied to date. The remainder of the 6-O- and 3,6-di-O-substituted galactose units, together with the 3-O-substituted galactose residues, are present in a highly branched framework of β1,6- and β1,3-linked D-galactose

residues. Some idea of the distribution of β1,3- and β1,6-linkages within this galactan framework was obtained by linkage and methylation analysis of the degraded gum B, isolated after a Smith degradation of degraded gum A. Partial acid hydrolysis of degraded gum B gave both the β1,3- and β1,6-galactobioses. An examination of methylated degraded gum B indicated the presence of 2,3,4,6-tetra- (++), 2,4,6- (++++) and 2,3,4-tri- (+), and 2,6- (+) and 2,4-di- (++), and 2-O-methyl-D-galactose (+), and 2,3,4-tri-O-methyl-D-glucuronic acid (a trace). Since all the uronic acid residues in degraded gum A are present as terminal non-reducing units, the identification of a small amount of uronic acid residues in degraded gum B is probably explained by the incomplete oxidation of a few uronic acid units by periodate during the Smith degradation of degraded gum A. Undermethylation is probably responsible for the presence of some 2,6-di- and 2-O-methyl-D-galactose. The proportions of 2,3,4,6-tetra- and 2,4-di- to 2,4,6- and 2,3,4-tri-O-methyl-D-galactose is evidence that degraded gum B is composed of highly branched galactan fragments. The fact that these fragments are sufficiently large in molecular size to be retained by cellophane dialysis tubing suggests that there is a significant proportion of mutually β1,3-linked D-galactose residues in degraded gum A, which are resistant to Smith degradation. The occurrence of 2,4,6-tri-O-methyl-D-galactose as the main O-methyl sugar component obtained from methylated degraded gum B is evidence for a high proportion of 3-O-substituted D-galactose residues in

degraded gum B. The identification of some 2,3,4-tri-O-methyl-D-galactose from methylated degraded gum B indicates that VI is a possible structural fragment of degraded gum A.



where R = L-Araf...., D-Galp...., ...D-Galp...., or ....D-Galp....

VI

The galactan framework of A.arabica gum appears to be based on a branched structure of β1,3- and β1,6-linked D-galactose residues. Two possible structures may be proposed. In the first, a "main chain" or "backbone" of blocks of β1,3- and β1,6-linked D-galactose units carries short side chains of D-galactose residues containing both β1,3- and β1,6-linkages. The second structure is based on a dichotomously branched structure of β1,3- and β1,6-linked D-galactose residues. It is suggested that A.arabica gum molecules possess dichotomous branching and are based on galactan frameworks of this second type.

The oligosaccharides obtained from the diffusate of the mild acid hydrolysate of the whole gum give some idea of the possible modes of linking of units in the arabinose-containing side chains, which are attached to the galactan framework. Two arabinobioses were separated on thick paper. The first was identical on paper chromatography to 3-O-β-L-arabinopyranosyl-L-arabinose. Acid

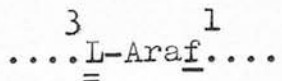
hydrolysis gave only arabinose and methylation analysis indicated that an arabinose unit was 3-O-substituted by an arabinopyranose residue. The disaccharide was characterised as its crystalline phenylosazone. The second arabinobiose had the same paper chromatographic mobility as 3-O-β-L-arabinofuranosyl-L-arabinose. Acid hydrolysis gave only arabinose and methylation analysis indicated that an arabinose unit was 3-O-substituted by an arabinofuranose residue. The fact that both arabinobioses are released by relatively mild acid hydrolysis conditions suggests that in each case the reducing ărabinose residue is initially present in the furanose form in the arabinose-containing side chains.

An examination of the O-methyl derivative of A.arabica gum indicated the presence of 2,3,5- and 2,3,4-tri-, and 2,5-, 3,5-, and 3,4-di-O-methyl-L-arabinose 2,4-di-O-methyl-D-galactose, and 2,3,4-tri-O-methyl-D-glucuronic acid together with small amounts of 2,3,4-tri-O-methyl-L-rhamnose, 4-O-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-, and 2-O-methyl-D-galactose. Small amounts of galactose and arabinose are not thought to be structurally significant. The structural significance of 4-O-methyl-L-arabinose and 2-O-methyl-D-galactose is also doubtful; they probably arise from either incomplete methylation or demethylation during hydrolysis. Although the presence of a high proportion of 2,4-di-O-methyl-D-galactose indicates that most of the D-galactose residues in A.arabica gum are 3,6-di-O-substituted, the identification of trace amounts of

2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-O-methyl-D-galactose is evidence for a few terminal non-reducing D-galactose residues and some 4-O-, 3-O-, and 6-O-substituted D-galactose units, respectively. The identification of 2,3,4-tri-O-methyl-D-glucuronic acid suggests that D-glucuronic acid and 4-O-methyl-D-glucuronic acid residues occur as terminal non-reducing units in the whole gum. The identification of trace amounts of 2,3,4-tri-O-methyl-L-rhamnose confirms the presence of small amounts of L-rhamnose residues as non-reducing end-groups in A.arabica gum (Appendix I; Anderson & Stoddart, 1965). However, the rhamnose content is so small that these residues must have little structural significance. The presence of 2,3,5- and 2,3,4-tri-O-methyl-L-arabinose indicates that the arabinose-containing side chains are terminated in some cases by L-arabinofuranose (VII) and in other cases by L-arabinopyranose residues (VIII). The identification of some 2,5-di-O-methyl-

VII VIII

L-arabinose is evidence for some 3-O-substituted L-arabinofuranose residues (IX) in the arabinose-containing side chains. The



IX

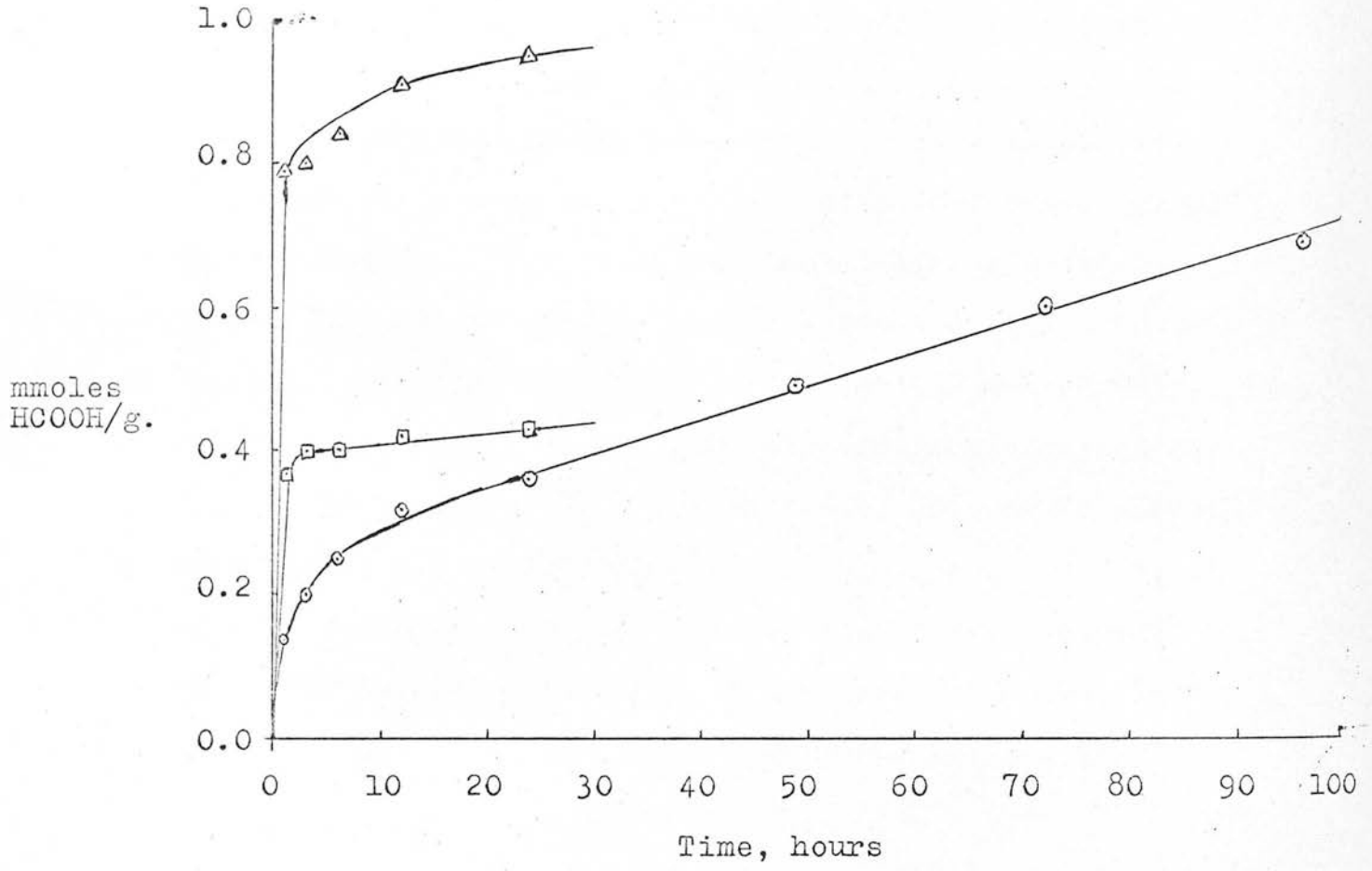


Figure 9. Formic acid released (mmoles/g.) with time (hours) on periodate oxidation of:-

(a) A.arabica gum (⊙)

(b) Polysaccharide I (⊡)

(c) Polysaccharide II (△)

major dimethyl ether of arabinose to be isolated and characterised was, however, the 3,5-di-O-methyl isomer. Previous to its isolation from methylated A.arabica gum, methylated A.pycnantha gum had been the only other O-methyl derivative of an Acacia gum to yield 3,5-di-O-methyl-L-arabinose (Nicolson, 1959; Aspinall et al., 1959). The O-methyl derivatives of A.nubica gum (Cree, 1966) and of A.drepanolobium gum (Dea, 1966) are now known to yield this di-O-methyl-L-arabinose. Its presence in methylated A.arabica gum suggests that there is a proportion of 2-O-substituted L-arabinofuranose residues (X) in the arabinose-containing side chains. If the small amounts of 3,4-di-O-methyl-L-arabinose do not arise from undermethylation or from demethylation, then there must be a small proportion of 2-O-substituted L-arabinopyranose residues (XI) in the arabinose-containing side chains as well. Methylation evidence for the

X XI

whole gum and for degraded gum A suggests that the arabinose-containing side chains are attached to the galactan framework at the C-3 and C-6 positions of certain D-galactose residues.

A sample of A.arabica gum was submitted to four successive Smith degradations. Figure 9, which shows a plot of the amount of formic acid released with time on periodate oxidation of A.arabica gum and polysaccharides I and II, shows that A.

arabica gum is susceptible to over-oxidation. The presence of some terminal non-reducing D-glucuronic acid residues in *A.arabica* gum may explain its susceptibility to over-oxidation (Huebner *et al.*, 1945; Hirst & Jones, 1955). The results of the Smith degradation experiments are summarised in Table 7. The percentage yields of Smith-degraded products are low compared with those obtained during successive Smith degradations on *A.senegal* gum. Molecular-sieve chromatography (Figure 6) indicates that there is pronounced cleavage of the macromolecule during successive Smith degradations; this cleavage of internal D-galactose residues is particularly drastic during the second and subsequent Smith degradations. The loss of a large number of small fragments during dialysis probably accounts for the low percentage yields of polysaccharides III and IV. The complete fragmentation of the macromolecule during successive Smith degradations shows that long chains of periodate-resistant β1,3-linked D-galactose residues are not such an important structural feature of *A.arabica* gum as they are of *A.senegal* gum (Chapter III). In *A.arabica* gum, blocks of periodate-immune β1,3-linked D-galactose units must be interspersed by blocks of periodate-vulnerable β1,6-linked D-galactose residues in a highly branched galactan framework. Fragmentation of this dichotomously branched galactan framework, which contains 6-O-substituted D-galactose residues, is to be expected as the arabinose-containing side chains are progressively removed from the C-3 positions of 6-O-substituted D-galactose units.

Table 7

Summary of results of Smith degradation experiments

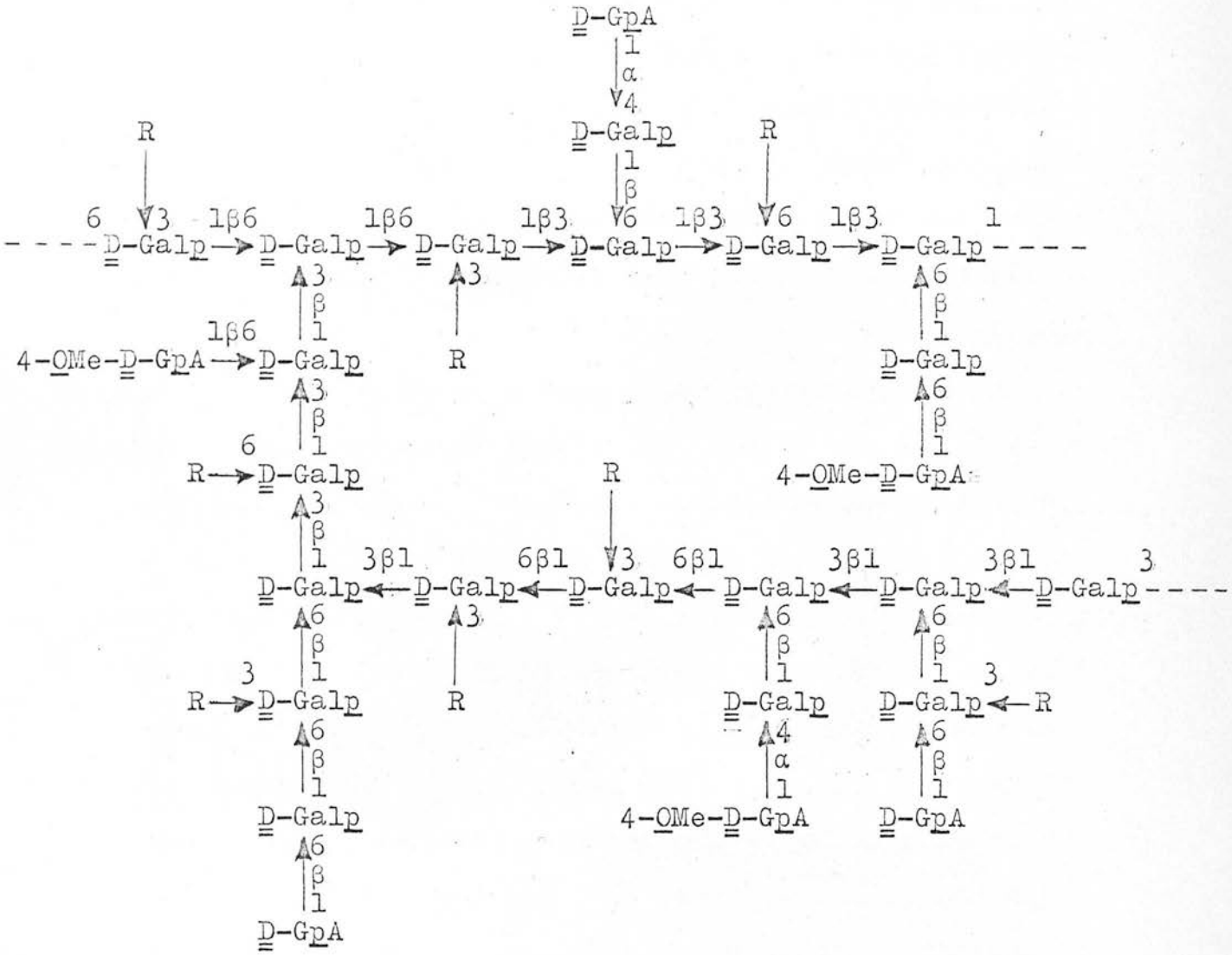
	Yield,%	$\bar{M}_n$	Gal,%	Ara,%	$[\alpha]_D$
A.arabica gum	-	>500,000	32	57	+112°
Polysaccharide I	59	>500,000	41	59	+63°
Polysaccharide II	44	-	50	50	+51°
Polysaccharide III	17	<2,000	72	28	+46°
Polysaccharide IV	2,5	-	-	-	-

The percentage values for galactose and arabinose, shown in Table 7, were determined by quantitative gas-liquid partition chromatography of their O-trimethylsilyl derivatives (Ludlow et al., 1966) as well as by the phenol sulphuric acid method (Dubois et al., 1956) after elution of the separated sugars from thick paper. Values obtained by the two methods agreed to within ±6%. Four successive Smith degradations were required to remove all the arabinose residues from A.senegal gum (Chapter III); in contrast, arabinose residues were not eliminated from the product (polysaccharide IV) obtained from A.arabica gum after four successive Smith degradations. This means that the arabinose-containing side chains in A.arabica gum contain more arabinose residues on the average than are present in the arabinose-containing side chains of A.senegal gum. This observation is predictable from the fact that A.arabica gum in contrast to

A.senegal gum contains more arabinose than galactose residues. During successive Smith degradations the specific rotation decreases from +112° for A.arabica gum to +46° for polysaccharide III. Linkages associated with the arabinose-containing side chains and their attachment to the galactan framework may be partly responsible for the high positive specific rotation of A.arabica gum.

On methanolysis, the O-methyl derivative of polysaccharides I, II, III, and IV gave the methyl glycosides of 2,3,5-tri-, 2,5- and 3,5-di-O-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-O-methyl-D-galactose. The identification of some 2,5- and 3,5-di-O-methyl-L-arabinose from methylated polysaccharide IV indicates that some of the arabinose-containing side chains in A.arabica gum must contain at least six mutually 1,3- and/or 1,2-linked L-arabinose units. The absence of any evidence for arabinopyranose residues in polysaccharide I confirms that the 4-O-methyl-L-arabinose characterised from methylated A.arabica gum is not structurally significant.

The structural evidence strongly suggests that A.arabica gum molecules possess a dichotomously branched galactan framework to which is attached uronic acid residues and arabinose-containing side chains. The dichotomous branching of the galactan frameworks implies that not all the uronic acid and arabinose residues are to be found on the periphery of the gum molecules. A possible structural fragment from one of



R represents L-arabinopyranose- and L-arabinofuranose- containing side chains. Some of these side chains are at least six units long and contain ....L-Araf....$^{3}_{1}$, ...L-Araf....$^{2}_{1}$, and ....L-Arap....$^{2}_{1}$ residues. They may be terminated by either L-Araf....$_{1}$ or L-Arap....$_{1}$ residues.

Figure 10. Possible structural fragment in Acacia arabica gum.

these gum molecules is represented by the polysaccharide array shown in Figure 10, and by the photograph of a model shown on Plate I. The galactan framework of A.arabica gum is more highly branched than the galactan framework of A.senegal gum, and the arabinose-containing side chains are longer in A.arabica gum than in A.senegal gum. These observations imply that the molecules of A.arabica gum have a much more compact structure than those of A.senegal gum. This conclusion is supported by the results of physical measurements made on solutions of the two gums. The fact that A.arabica gum has a lower limiting viscosity number than A.senegal gum, despite its higher weight-average molecular-weight, is evidence that A.arabica gum molecules are more densely packed with sugar residues and consequently more compact than A.senegal gum molecules.

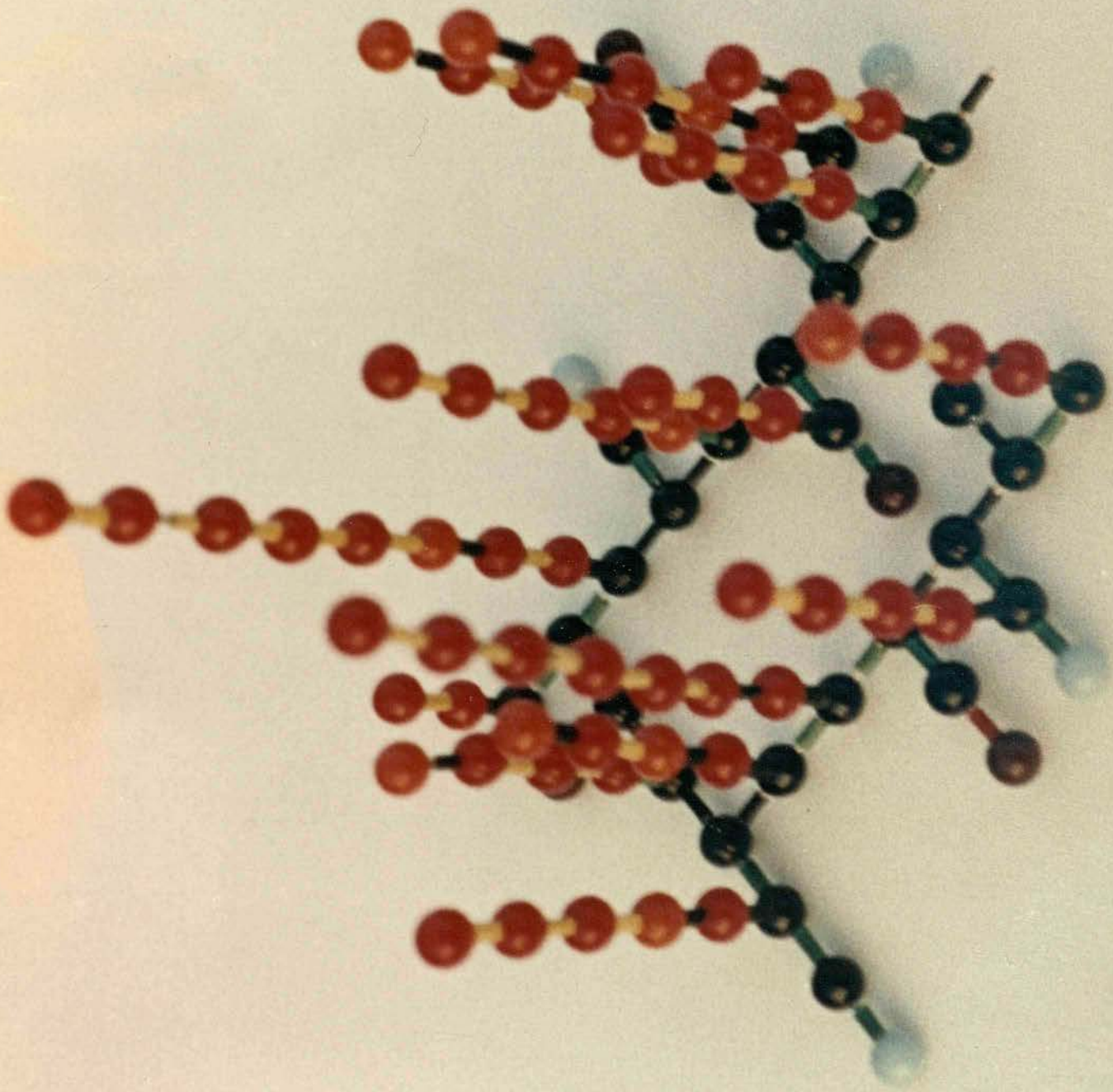


Plate I. This plate shows a colour photograph of a model, which represents the author's concept of how 100 sugar residues may be linked together in a structural fragment from a molecule of A.arabica gum. Each ball represents a sugar residue. The model portrays one possible assembly of sugar residues.

Key

Balls	Sugar residues	Bonds	Linkages
Black	D-Galp	Black	1,3
Blue	D-GpA	Green	1,6
Brown	4-OMe-D-GpA	Yellow	1,2
Orange	L-Arap	Red	α1,4
Red	L-Araf		

CHAPTER V

GENERAL SUMMARY AND CONCLUSIONS

The results of a re-investigation of A.senegal gum have led to the rejection of the idea that its molecular structure is necessarily based on a "main chain" or "backbone" of β1,3-linked D-galactose residues. The concept that the A.senegal gum molecule contains a "main chain" or "backbone," to which are attached short side chains, implies that there is one chain in the macromolecule which is unique in being very much longer than the others. Unequivocal evidence for the occurrence of such chains in A.senegal gum molecules does not exist. In the case of A.arabica gum, all the evidence from structural investigations, and from the solution behaviour of the gum favours a dichotomously branched galactan framework for the basal units of the macromolecules. These findings suggest that it is no longer meaningful to interpret the structural features of these gums in terms of average repeating units. The results, however, may have a wider implication and significance. On re-examination, many plant gums (Smith & Montgomery, 1959) of the substituted arabinogalactan type, and perhaps some of the arabinogalactans themselves (Timell, 1965), may be shown to exhibit dichotomous branching within their galactan frameworks.

Although structural work has been carried out on gums from other Acacia species, including A.mollissima (Stephen, 1951;

Young, 1963), A.pycnantha (Hirst & Perlin, 1954; Aspinall, Hirst & Nicolson, 1959), A.karroo (Charlson, Nunn & Stephen, 1955a), A.cyanophylla (Charlson, Nunn & Stephen, 1955b), A.catechu (Hulyalkar, Ingle & Bhide, 1956, 1959), A.sundra (Mukherjee & Shrivastava, 1957, 1958, 1959; Shrivastava, 1962), A.seyal (Herbich, 1963), A.nilotica (Karamalla, 1965; Anderson & Karamalla, 1966b), A.nubica (Cree, 1966) and A.laeta (Smith, 1966), the investigations reported in this thesis represent the first exploratory steps towards interpreting molecular structures of Acacia gums at the molecular level.

APPENDIX I

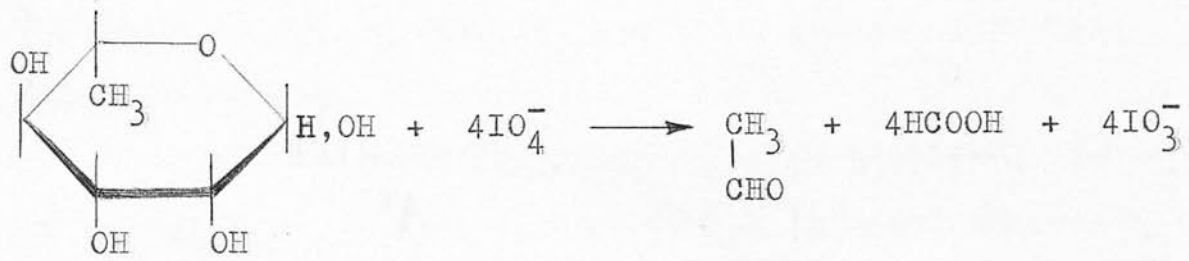
A VAPOUR-PHASE INFRARED METHOD FOR THE ESTIMATION OF RHAMNOSE

I.1) Introduction

During the course of the investigations on the *Acacia* gum exudates mentioned in Chapters III and IV, it became obvious that a specific and sensitive method for determining rhamnose was required. Although several methods for the determination of rhamnose, and other 6-deoxyhexoses, have been proposed, each has been deficient in either specificity or sensitivity. Marshall & Norris (1937*a*,*b*) used a procedure based on the principle that 6-deoxyhexoses, on distillation with hydrochloric acid (13%, w/v), yield methylfurfuraldehyde which can be determined either gravimetrically or titrimetrically. The yield of methylfurfuraldehyde is not quantitative, however, and empirical correction factors have to be introduced. Dische & Shettles (1948, 1951) described a spectrophotometric procedure for determining 6-deoxyhexoses; this involved heating with concentrated sulphuric acid and the development of a specific colour with cysteine hydrochloride. More recently, Toennies & Kolb (1964) have used anthrone, instead of cysteine hydrochloride, for development of a specific coloured derivative; in addition, these investigators found that values for rhamnose contents, obtained by their anthrone procedure, were 14-40% lower than those

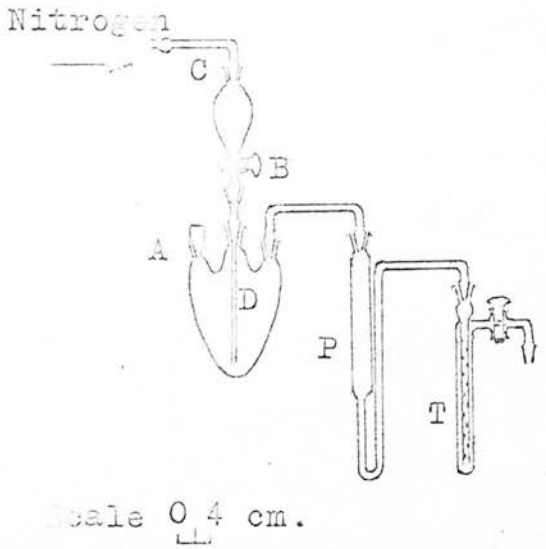
obtained using the cysteine hydrochloride method. The lack of agreement between the results given by these two independent colorimetric procedures does not serve as a recommendation for either; other methods for determining rhamnose had to be reviewed.

An assessment of the procedures available indicated that a method based on the oxidation of rhamnose with periodate to release acetaldehyde (Nicolet & Shinn, 1941; Cameron, Ross & Percival, 1948; Black, Cornhill, Dewar, Percival & Ross, 1950) offered the greatest chance of success. If periodate oxidation of rhamnose occurs quantitatively to give acetaldehyde according to the equation,



L-rhamnopyranose

the task becomes one of determining this aldehyde in complex aqueous reaction mixtures. Acetaldehyde may be removed from such reaction mixtures by aeration; this aspiration technique was apparently first used by Clausen (1922); it has been used frequently since (Block & Bolling, 1939; Nicolet & Shinn, 1941; Rees, 1946; Cameron et al., 1948; Black et al., 1950; Cox, 1952; Tompsett, 1958). Thereafter, acetaldehyde is usually trapped in aqueous solution as its bisulphite complex and determined



A = Inlet for hydrolysate

B = Tap to start reaction

C = Nitrogen inlet

D = Inlet tube

P = Pre-trap for water vapour

T = Trap for acetaldehyde

Figure 1. The assembled apparatus.

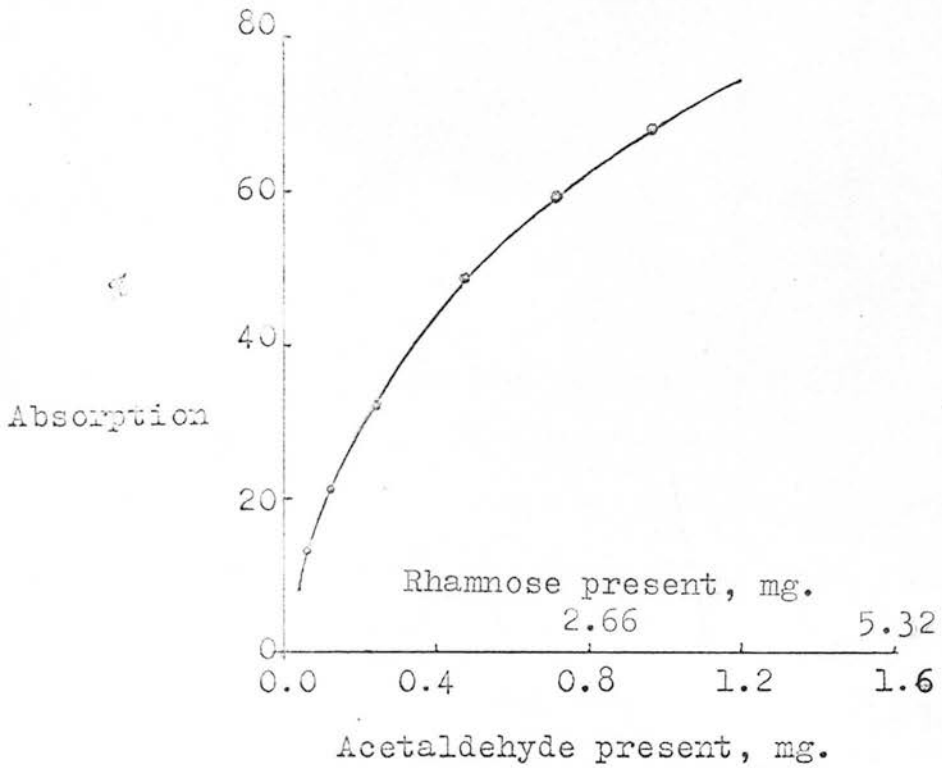


Figure 2. Calibration curve for acetaldehyde.

either titrimetrically (Nicolet & Shinn, 1941; Cameron *et al.*, 1948) or colorimetrically (Black *et al.*, 1950). As before, there is disagreement between results obtained using different methods of assay; for this reason, the necessity for a more selective and sensitive method for determining acetaldehyde has recently received comment (Sawicki, 1962). Although gas-liquid partition chromatography (Hoffman, Barboriak & Hardman, 1964) offers a more sensitive method of determinating acetaldehyde, vapour-phase infrared spectroscopy was preferred in the present investigation on account of its greater specificity.

I.2) Materials and Methods

Acetaldehyde was twice redistilled. Rhamnose monohydrate was obtained from T. Kerfoot & Co. Ltd., Vale of Bardsley, Lancashire.

Gas-cell calibration (Anderson & Duncan, 1958; Anderson, 1959) was based on the strong carbonyl absorption given by acetaldehyde vapour at 1750 $cm.^{-1}$. Calibration curves were constructed, under standardised spectrometer operating conditions, by using the stoppered weighing-bottle technique described by Anderson & Duncan (1960) for volatile liquids.

The apparatus required for aspirating acetaldehyde from aqueous reaction mixtures at room temperature is shown in Figure 1. The outlet from the three-necked flask (100 ml. capacity) is connected, *via* a wide-bore U-tube, P, to a liquid nitrogen trap, T, details of which have been described by Anderson & Duncan (1960). The U-tube is immersed in a Dewar flask (1 pint capacity) containing

solid carbon dioxide in acetone; this serves as a pre-trap for water vapour without retaining any acetaldehyde. The wide-bore entry shown for pre-trap P is necessary to prevent deposition of ice from blocking the flow system. The inlet tube, D, reaches to the bottom of the flask and carries a dropping funnel (25 ml. capacity). Cylinder nitrogen, at a flow-rate of 300-350 ml. per minute, is used for aspirating the acetaldehyde.

The following reaction procedure was adopted. Aliquots (1-5 ml.) of standard rhamnose solutions, or of neutralised polysaccharide hydrolysates containing rhamnose, were transferred to the three-necked flask via neck A. Potassium arsenite solution, N (1 ml.), 5 ml. of 0.133M-phosphate buffer, pH 7.0, and sufficient water to make the final volume up to 17 ml. were added to the flask. Sodium metaperiodate solution (0.5M, 3 ml.) was pipetted into the dropping funnel and the reaction started by opening tap B and simultaneously inserting the nitrogen delivery tube into socket C; the periodate solution was thus introduced into the flask from the dropping funnel under pressure from the nitrogen. A nitrogen flow-rate of 300-350 ml. per minute was maintained for 2 hours. During this time a considerable amount of ice was deposited in pre-trap P, but there was no retention of acetaldehyde, which was collected in trap T. At the end of the aspiration period, the acetaldehyde was transferred from trap T to a gas-cell of suitable sensitivity (Anderson & Duncan, 1958) for quantitative determination using the technique described by Anderson (1959). Immersion of trap T

in hot water (60° - 70°C.) vaporised the acetaldehyde.

Polysaccharide hydrolyses were carried out as follows. A suitable weight of polysaccharide (approximately 200 mg. for a rhamnose content of 1% and approximately 50 mg. for a rhamnose content of 10%) was hydrolysed on a boiling water bath with 2N-sulphuric acid (12 ml.) for 2.5 hours. The hydrolysate was then transferred with careful rinsing to a 25 ml. volumetric flask and neutralised (methyl orange indicator) with 5N-sodium hydroxide solution; the volume in the flask was finally adjusted to 25 ml. with water. Portions (3, 4, or 5 ml.) were then placed in the three-necked flask for periodate oxidation. Smaller samples of polysaccharide (e.g. 5-10 mg. for a rhamnose content of 10%) could be hydrolysed, neutralised and transferred directly to the three-necked flask for single determinations.

I.3) Results and Discussion

During the initial development of the method, it was necessary to use two gas cells of different sensitivities; each had its own calibration curve; a relatively insensitive cell, fitted with sodium chloride windows, was used when experiments (rate of aspiration, etc.) directly involving weighed amounts of acetaldehyde were in progress, and a cell of standard sensitivity (length, 12.5 cm.; internal volume, 31 ml., fitted with windows of "Irtran-4" glass, (Messrs. Kodak Ltd., Kirkby, Liverpool) was used for the much smaller amounts of acetaldehyde evolved from suitable weights of rhamnose in standard solutions or polysaccharide hydrolysates.

Amounts of acetaldehyde of up to 1 mg. were quantitatively aspirated from 21 ml. of water within 90 mins. at nitrogen flow-rates of 300-350 ml. per min. In subsequent determinations an aspiration period of 120 mins. was allowed. Traces of carbon dioxide and water vapour did not interfere with the spectroscopic determination of acetaldehyde vapour. For individual weights of rhamnose monohydrate in the range 1.5-6.0 mg., weights of acetaldehyde corresponding to recoveries of rhamnose monohydrate of between 96 and 104% were obtained by the procedure outlined in the previous section. Similar recoveries were obtained for fucose, and for rhamnose monohydrate, to which had been added equal weights of arabinose, galactose and glucuronic acid. Since considerable amounts of formic acid are released on periodate oxidation of carbohydrate material, the reaction medium must be adequately buffered to prevent an increase in acidity as oxidation proceeds. The most satisfactory results were obtained when a pH of 7.0 was maintained with a phosphate buffer system (Rees, 1946). The bicarbonate buffer, recommended by Nicolet & Shinn (1941), gives copious evolution of carbon dioxide; deposition of solid carbon dioxide in the liquid nitrogen trap, T, blocks the flow system within a few minutes; for this reason, a bicarbonate buffer could not be employed. Arsenite is included in the reaction mixture to reduce any excess of periodate; the reaction of arsenite with periodate is catalysed by iodide ion and has a relatively low rate when compared with that of the oxidation of rhamnose by periodate

(Bobbitt, 1956); the inclusion of arsenite in the reaction mixture minimises oxidation of acetaldehyde by periodate. The calibration curve shown in Figure 2 was constructed using aliquots (0.50-5.00 ml.) of a standard solution made by dissolving rhamnose monohydrate (400 mg.) in water (500 ml.). The "rhamnose" scale on the abscissa is expressed in mg. units of the anhydro-sugar; all values subsequently quoted for rhamnose contents are for the anhydro-form.

Nicolet & Shinn (1941), Cameron et al. (1948) and Black et al. (1950) added alanine to their reaction mixtures, presumably to trap formaldehyde (released on periodate oxidation of a pentose or hexose) as dimethylolalanine, and thus ensure that formaldehyde was not aspirated along with acetaldehyde. Fleury et al. (1949) reported that periodate oxidation of alanine to acetaldehyde at room temperature occurs to a small extent; the optimum pH for this reaction was shown to be 8.0; periodate oxidation was claimed to be negligible at pH 7.6. In addition, Nicolet & Shinn (1939) reported that alanine undergoes reaction with periodate at a rate which is only 0.1% of that of the β-hydroxy amino acids, serine and threoine. Moreover, Black et al. (1950) found that high results may be obtained using the method of Nicolet & Shinn (1941), and suggested that an aldehyde other than acetaldehyde or formaldehyde is being liberated. Vapour-phase infrared spectroscopy showed that no aldehyde other than acetaldehyde was liberated, when the method of Nicolet & Shinn (1941), involving the addition of alanine to

reaction mixtures, was used; however, high results were obtained, when alanine was added. Thus, it appeared that the addition of comparatively large amounts of alanine (200 mg.) might be responsible for "over-production" of acetaldehyde. This was confirmed. In experiments with alanine (micro-analytical reagent grade), under the reaction conditions described, slow oxidation of the amino acid was shown to occur; samples (200 mg.) of alanine produced 0.30 mg. of acetaldehyde (average of five determinations) in 2 hours. Although aspiration for this period of time was allowed, the effective duration of the periodate oxidation reaction, which was arsenite controlled, was undoubtedly much less. Similar results were obtained with two other specimens of alanine obtained from other sources. These results imply that, if alanine (200 mg.) is added to rhamnose (1.64 mg.) the recovery of acetaldehyde, after aspiration for 2 hours, will be 0.44 mg. (from rhamnose) plus 0.30 mg. (from alanine). Therefore, the addition of alanine should not be made. Moreover, under the reaction conditions described, vapour-phase infrared spectroscopy showed that volatilisation of formaldehyde did not occur in the absence of alanine.

Since rhamnose must be available in the free form for periodate oxidation to yield acetaldehyde, polysaccharides containing rhamnose must be hydrolysed. The hydrolysis conditions required by two Acacia gum exudates (from A.senegal and A.karroo), differing widely in rhamnose contents, were investigated; it was found that hydrolysis on a boiling-water bath with both

N-sulphuric acid and 2N-sulphuric acid followed first order reaction kinetics; the times required for complete hydrolysis of rhamnopyranosidic bonds were 6 hours, and 2.5 hours, respectively. The decomposition of rhamnose was studied under these conditions, and although no detectable decomposition occurred with either system in the times stated, hydrolysis with 2N acid for 2.5 hours was preferred for Acacia specimens.

Determinations on a sample (different from that used in Chapter III) of A.senegal gum gave a rhamnose content of 11.6%. Determinations on the exudate gum from A.karroo gave a rhamnose content of 1.8%; this result substantiates the provisional value reported by Charlson, Nunn & Stephen (1955a), who found this amount "too small to estimate with accuracy." Results obtained for some other Acacia specimens are given in Table 1. The agreement between the replicates indicates the confidence that may be shown in determinations made on Acacia gum exudates of extremely low rhamnose contents. When the amount of rhamnose present in the gum exudate is extremely small, a useful technique is to transfer a known weight of rhamnose to the reaction flask along with the portion of hydrolysate. The rhamnose content of the Acacia specimen may then be calculated from the apparent excess in the recovery of rhamnose. This technique allows spectroscopic measurement to be made with respect to the most sensitive portion of the calibration curve; its use has been described in greater detail by Anderson & Zaidi (1964).

Table 1

The rhamnose content of some *Acacia* species

Acacia Species	Weight of gum sample in portion taken, mg.	Weight of rhamnose added, (a), mg.	Weight of acetaldehyde found, (b) mg.	Total recovery of rhamnose, ($c=b \times \frac{146}{44}$) mg.	Weight of rhamnose in gum sample, (c-a) mg.	Rhamnose* present, %
A.nilotica	40.4	0.34	0.148	0.49	0.15	0.4
	40.4	0.34	0.145	0.48	0.14	0.4
A.arabica	41.6	0.34	0.148	0.49	0.15	0.4
	41.6	0.34	0.150	0.50	0.16	0.4
A.nubica	40.0	0.34	0.166	0.55	0.21	0.5
	40.0	0.34	0.169	0.56	0.22	0.5
A.drepanolobium	48.3	Nil	0.129	0.43	0.43	0.9
	48.3	Nil	0.137	0.45	0.45	0.9
A.campylacantha	12.5	Nil	0.236	0.78	0.78	6.2
	12.5	Nil	0.231	0.77	0.77	6.2

* Expressed as the anhydride.

The procedure gives an overall accuracy greater than has been available to date. Furthermore, the method gives reliable results at lower levels of rhamnose content in Acacia gum exudates than could previously be determined.

The results of these investigations were presented in Nottingham at a conference held under the auspices of the Society for Analytical Chemistry (Anderson & Stoddart, 1965). Since then, Bhattacharyya & Aminoff (1966) have published a spectrophotometric modification of an earlier Conway diffusion procedure (Aminoff & Morgan, 1951) for the determination of acetaldehyde after periodate oxidation of 6-deoxyhexoses.

APPENDIX II

ZONE ELECTROPHORETIC STUDIES ON *ACACIA* GUMS

II.1) Introduction

The results of several investigations on the electrophoretic properties of *Acacia* gums have been reported in the literature. Gum arabic has been shown (*cf.* Hirst & Jones, 1955) to be "electrophoretically homogeneous" in a Tiselius apparatus using a borate buffer. Joubert (1954) has examined the electrophoretic properties of *A.cyanophylla* gum and gum arabic under similar conditions; he observed considerable differences between the descending and ascending electrophoretic patterns on free-boundary electrophoresis, and attributed these to "electrophoretic heterogeneity." The exudate gums from *A.arabicum*, *A.pycnantha*, *A.senegal*, and *A.seyal* were found (Lewis & Smith, 1957) to be "heterogeneous" on glass-fibre electrophoresis in 2N-sodium hydroxide solution, whereas black wattle gum from *A.decurrens* appeared to be "homogeneous." Two samples of *A.pycnantha* gum were examined electrophoretically by Aspinall, Hirst & Nicolson (1959) on glass-fibre paper using 2N-potassium hydroxide solution as electrolyte (Nicolson, 1959). One sample was found to be "essentially homogeneous," while the other sample was shown to contain two components. In contrast, Anderson & Herbich (1963) observed no distinct separation of components, when *A.seyal* gum was examined

electrophoretically on glass-fibre paper using 2N-sodium hydroxide solution as electrolyte.

Although glass-fibre paper has been recommended (Bourne, Foster & Grant, 1956) as a stabilising medium for polysaccharide electrophoresis, because of the ease of detection of polysaccharide bands (Lewis & Smith, 1957), and because complexes between polysaccharides and the fibre are avoided, zone electrophoresis has also been carried out on filter paper (Rienits, 1953; Geldmacher-Mallinckrodt & Weinland, 1953; Preece & Hobkirk, 1955; Geldmacher-Mallinckrodt & Traxler, 1961), and on cellulose acetate (Håkkinen, Hartiala & Terho, 1965). The detection of polysaccharides after migration on filter paper has presented a difficult problem; although Rienits (1953) has reported that the periodic acid-fuchsin stain is unsatisfactory with filter paper, Geldmacher-Mallinckrodt & Traxler (1961) have used it with success. Aniline oxalate has been used (Preece & Hobkirk, 1955) to detect polysaccharide bands after migration on filter paper, and "alcian blue" (Håkkinen, Hartiala & Terho, 1965) to stain acidic polysaccharides after migration on cellulose acetate.

II.2) Results and Discussion

In view of the different results obtained for *Acacia* gums, after electrophoresis on glass-fibre paper, it was decided to investigate the possibility of using filter paper and cellulose acetate as stabilising media. Since the molecules of *Acacia*

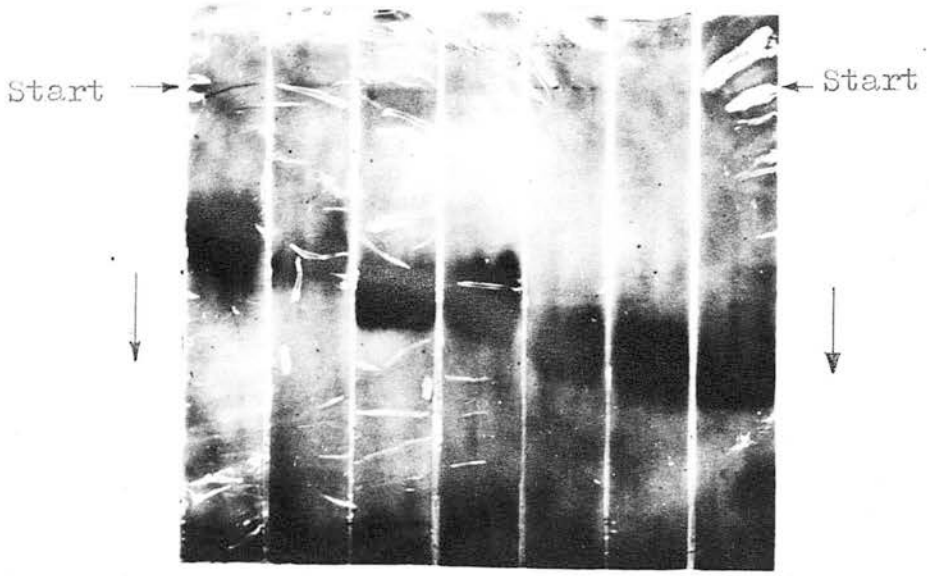


Plate I. This plate shows the migration behaviour of the gums from seven different Acacia species (listed below) on Whatman No. 1 paper in 0.1M-ammonium carbonate buffer (pH, 8.9) at 5 volts/cm. After electrophoresis for 22 hours, polysaccharide bands were located by the periodic acid-fuchsin stain and the wet paper strips photographed between sheets of cellophane. The light reflection properties of the cellophane sheet account for the spurious marks on the plate.

	Species	Uronic acid content, %.
a	A.pycnantha	5
b	A.arabica	10
c	A.mearnsii	8
d	A.campylacantha	9
e	A.karroo	12
f	A.seyal	14
g	A.senegal	19

gum will be negatively charged when the carboxyl groups, associated with the uronic acid residues, are ionised, or even partially ionised, the use of 2N-sodium hydroxide solution in order to ionise alcohol groups (Lewis & Smith, 1957) would appear to be unnecessary. Moreover under these alkaline conditions, some degradation of polysaccharide gums may occur. Thus, in the experiments described in this section, a 0.1M-ammonium carbonate buffer (pH, 8.9) and a 0.1M-acetate buffer (pH, 4.7) were used. (I wish to thank Dr. D. Gross for recommending an ammonium carbonate buffer system for use with acidic polysaccharides). After migration, polysaccharide bands were located on both filter paper and cellulose acetate by using a modification of the periodic acid-fuchsin stain.

Plate I shows the migration behaviour of the gums from seven different Acacia species [viz., A.pycnantha, A.arabica, A.mearnsii (syn A.decurrens), A.campylacantha, A.karroo, A.seyal, and A.senegal] on filter paper in 0.1M-ammonium carbonate buffer (pH, 8.9). Plate II shows the migration behaviour of the same seven Acacia species on cellulose acetate in the same buffer system. In all cases the gums migrated towards the anode as single bands. The rates of migration of the gums also appear to bear some relationship to their uronic acid contents; in general, the higher the uronic acid content, the faster is the rate of migration towards the anode at the same applied potential. Thus, the mobility is dependent, amongst other variables (which will include molecular size), on the number of

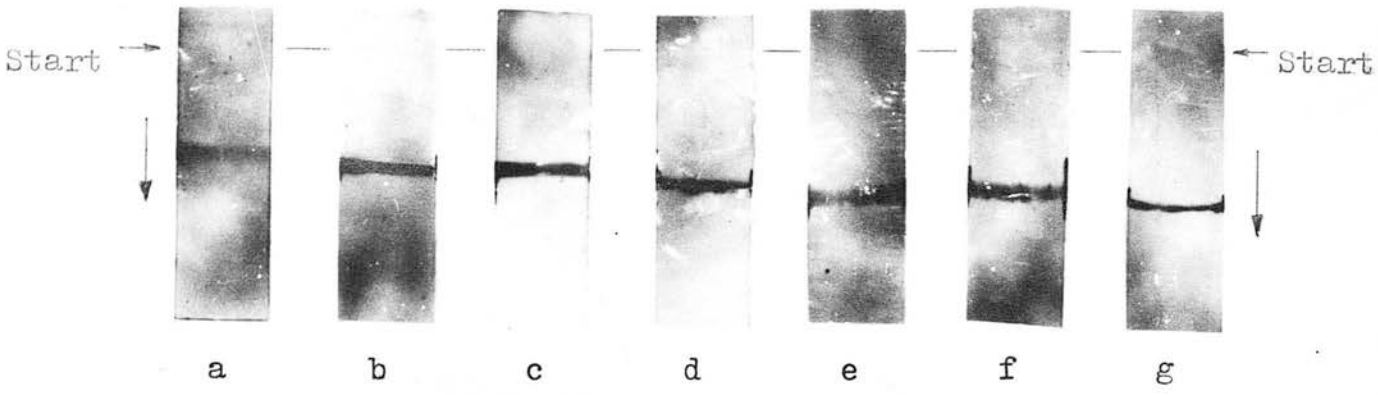


Plate II. This plate shows the migration behaviour of the gums from seven different Acacia species (listed below) on cellulose acatate film in 0.1M-ammonium carbonate buffer (pH, 8.9) at 18.8 volts/cm. After electrophoresis for 2.5 hours, polysaccharide bands were located by the periodic acid-fuchsin stain and the wet cellulose acetate strips photographed between sheets of cellophane. The light reflection properties of the cellophane sheet account for the spurious marks on the plate.

	Species	Uronic acid content, %.
a	A.pycnantha	5
b	A.arabica	10
c	A.mearnsii	8
d	A.campylacantha	9
e	A.karroo	12
f	A.seyal	14
g	A.senegal	19

carboxyl groups, and hence the uronic acid content of the gum. The seven Acacia species mentioned above also migrated as single bands on electrophoresis in 0.1M-acetate buffer (pH, 4.7) on cellulose acetate; the bands, however, were much broader than those obtained after electrophoresis in 0.1M-ammonium carbonate buffer at pH, 8.9. Other Acacia gums, which were shown to migrate as single bands in 0.1M-ammonium carbonate buffer (pH, 8.9) on cellulose acetate, include A.adansoniana, A.laeta, A.multijuga, A.nilotica, A.nubica, and A.tortilis.

The results obtained with gum samples from various Acacia species suggest that cellulose acetate could prove to be a useful stabilising medium for zone electrophoresis of polysaccharides.

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The analytical importance of the methoxyl content of *Acacia* gum exudates

SIR,

Methoxyl groups occur frequently in plant gums as 4-methoxyglucuronic acid (*e.g.*, in *Albizzia*[1] and *Khaya*[2] species) or as ester groups (*e.g.*, in *Sterculia*[3] and *Astragalus*[4] gums). To date, however, the possibility of the presence of methoxyl groups in *Acacia* gum exudates appears largely to have been ignored, *e.g.*, in studies of *A. senegal*,[5] *A. pycnantha*,[6] *A. karroo*,[7] *A. cyanophylla*[8] and *A. sundra*,[9] although Stephen reported (without comment) a value of 0·35% for *A. mollissima*,[10] and Hulyalkar *et al.* found no methoxyl content in *A. catechu*.[11]

Recently, Anderson and Herbich observed[12] that the methoxyl content of a number of nodules of the gum from *A. seyal* ranged from 0·7–1·3%, and this has led us to analyse specimens of the gum from 12 further *Acacia* species, not hitherto studied chemically. We have also re-investigated three different specimens of *A. senegal syn. Verek* (gum arabic) and a sample of *A. karroo*. An infrared method,[13] specific for methoxyl groups, was used to analyse purified samples, prepared from authenticated single nodules of each species by electrodialysis so that artifacts arising from solvent retention[14] could not occur (*cf.* ref. 1).

The results shown in Table I indicate that the presence of methoxyl groups in *Acacia* gums is a more general occurrence than hitherto believed. In addition, the range of values found (0·75 to 1·44%) for 9 nodules of *A. nilotica* substantiates recent evidence[12,15] for inter-nodule variation in the composition of plant gums.

The viscosity of samples of gum tragacanth and of pectins is known[16] to be related to their methoxyl content. The limiting flow-time numbers for some of our *Acacia* samples, determined under standardised conditions, are also shown in the Table: a plot of methoxyl content *versus* limiting flow-time number gives a smooth curve.

TABLE I

Acacia species	Methoxyl, %*	Limiting flow-time number†
A. giraffae Burch	2·40	
A. nilotica (L.) Willd. ex Del.	1·14[a]	10·4
A. mellifera (Vahl) Benth.	1·06	
A. seyal Del.	1·02[b]	12·1
A. seyal Del. var. fistula	0·90	
A. arabica (Lam) *Willd.*	0·88	12·5
A. tortilis (Forsk.) *Hayne.*	0·57	
A. mearnsii De Wild.	0·45	
A. campylacantha Hochst. ex A. Rich.	0·42	16·0
A. drepanolobium Harms ex Sjöstedt.	0·40	16·6
A. senegal (L.) *Willd.*	0·36[c]	19·2
A. dealbata Link.	0·35	21·5
A. laeta R. Br. ex Benth.	0·33	23·0
A. nubica Benth.	0·15	
A. karroo Hayne.	0·13	

* Electro-dialysed, freeze-dried samples, corrected for trace residual moisture and ash content.
† In aq. 4% NaCl solution at 25°.
[a] Average of results for 9 nodules (range 0·75–1·44%).
[b] Average of results for 6 nodules (range 0·70–1·30%).
[c] Average of results for 3 nodules (range 0·34–0·37%).

It is therefore suggested that the methoxyl content of *Acacia* gums has some structural significance, and that greater analytical attention should be given to this in future studies. We do not subscribe to the view, recently expressed[17] in a study of gum Jeol, that a methoxyl content of 0·51% can readily be dismissed as being very low and not structurally significant.

It is of interest that our re-examination of *A. senegal* and *A. karroo* has revealed the presence of methoxyl groups. Re-examination of other species, *e.g.*, *A. pycnantha*, may well provide an explanation for the complex behaviour observed[6] during examination of the aldobiuronic acid fraction.

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An Infrared Method for the Determination of Small Amounts of Acetaldehyde in Aqueous Solution*

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A specific and accurate method is described for determining acetaldehyde in aqueous solutions on the microgram scale. After quantitative aspiration from solution with a rapid flow of nitrogen, the acetaldehyde is determined by vapour-phase infrared spectroscopy. Other lower aldehydes, acids and alcohols in the aqueous solution do not interfere. The method is therefore particularly useful when used in conjunction with analytical procedures involving periodate oxidation, *e.g.*, the determination of methyl pentoses in carbohydrates, and of threonine in proteins.

As knowledge of the chemistry of natural products increases, there is always a need for more accurate and specific methods of analysis, so that consolidation, correction and refinement of earlier studies, often exploratory, can be made.[1]

For polysaccharides of both land and aquatic origin, investigations centred upon the methyl pentose sugars have given useful information about the presence of heterogeneity[2] and the nature of structural fragments.[3] Although several methods, *e.g.*, distillation as methyl furfural,[4] colorimetric[5] and titrimetric,[6] for determining methyl pentoses have been proposed, each has been deficient in either sensitivity, specificity or accuracy, with the result that some doubt has existed[7] about the significance of some observations.[8,9] Our immediate requirement, for studies on *Acacia* gum exudates, was for a method giving results to within $\pm 0{\cdot}1$ per cent. absolute at the 0 to 1 per cent. level of rhamnose, and $\pm 0{\cdot}2$ per cent. at the 1 to 15 per cent. level.

A review of the procedures available indicated that a method based on the periodate-oxidation release of acetaldehyde[10] offered the greatest chance of success. The task therefore involved finding a method for improving the existing methods for determining small amounts of acetaldehyde in complex aqueous reaction mixtures. The necessity for a more selective and sensitive method to be found has already received comment,[11] and several investigations with this aim have been reported recently.[12,13,14] Gas chromatography has also been used in a more original approach,[15] although the more specific (if slightly less sensitive) technique of infrared spectroscopy was, whenever possible, preferred for the present study. When it was realised that the accurate determination of rhamnose was also required in studies of bacterial metabolism,[16,17] and that the determination of threonine, as acetaldehyde, is of current importance in studies of proteins[18] and lipids,[19] it was decided to investigate the determination of acetaldehyde from a more general analytical viewpoint.

The method proposed involves the production of acetaldehyde from the protein or carbohydrate hydrolysate by periodate oxidation at pH 7·0, the removal of

* Part XIV of the series, "Applications of Infrared Spectroscopy."

the acetaldehyde from the aqueous solution by an aspiration technique (apparently first used by Clausen,[20] and subsequently used frequently[10,21,22,23]) and the determination of the acetaldehyde by the specific method of vapour-phase infrared spectroscopy. The flow-gas emerging from the reaction vessel is passed through a preliminary cold-trap (acetone - solid carbon dioxide) to remove water vapour; the acetaldehyde is then collected quantitatively in a trap immersed in liquid nitrogen and is subsequently transferred[24] as a vapour to a gas-cell for quantitative determination.

Experimental

Apparatus—

The apparatus required for aspirating acetaldehyde from aqueous reaction mixtures at room temperature is shown in Fig. 1. The outlet from the three-necked flask (100-ml capacity) is connected, *via* a wide-bore U-tube, to a liquid-nitrogen trap, details of which have already been described.[25] The U-tube is immersed in a

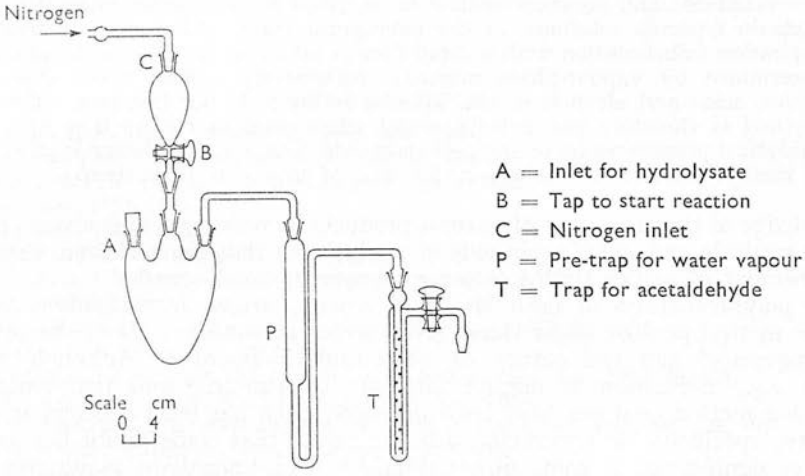

Fig. 1. The assembled apparatus

Dewar flask (1-pint capacity) containing solid carbon dioxide in acetone; this serves to trap the water vapour without retaining any of the acetaldehyde. Cylinder nitrogen, at a flow-rate of 300 to 350 ml per minute, is used for aspirating the acetaldehyde. The inlet tube reaches to the bottom of the flask, and carries a dropping funnel (25-ml capacity); at the start of a reaction, the periodate solution is introduced from this dropping funnel under pressure from the nitrogen.

Reagents—

Sodium m-*periodate solution*, 0·5 M.

Potassium arsenite solution, N.

Phosphate buffer solution, pH 7·0—A solution 0·0906 M with respect to disodium hydrogen phosphate and 0·0426 M with respect to potassium dihydrogen phosphate was used.

Acetaldehyde, twice redistilled.

Rhamnose monohydrate—Obtained from T. Kerfoot and Co. Ltd., Vale of Bardsley, Lancs.

DL-α-*Alanine*—M.A.R. grade.

Spectroscopic determination of acetaldehyde—

Acetaldehyde was transferred quantitatively from the liquid-nitrogen trap to a gas-cell of suitable sensitivity,[26] by the technique previously described.[24] Immersion of the cold-trap in hot water was sufficient to effect conversion of the acetaldehyde to the vapour-phase. The calibration is based on the strong carbonyl absorption given by acetaldehyde vapour at 1750 cm^{-1}. A typical calibration curve is shown in Fig. 2, and was constructed, under standardised spectrometer operating

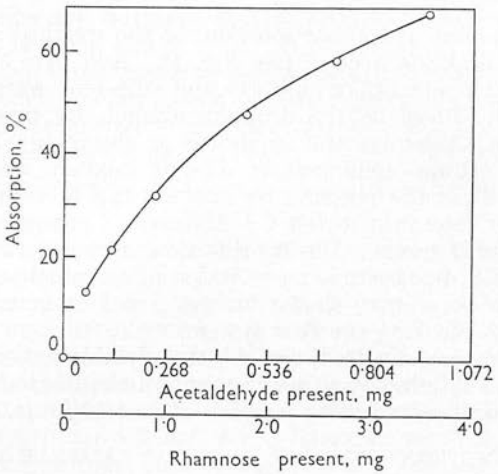

Fig. 2. Calibration curve for rhamnose in terms of acetaldehyde absorption at 1750 cm^{-1}

conditions, by using the stoppered weighing-bottle technique already described[25] for volatile liquids. During this investigation, it was necessary to use two gas-cells of different sensitivities, each having their respective calibration curves; a relatively insensitive cell was used when experiments (rate of aspiration, etc.) directly involving weighed amounts of acetaldehyde were in progress, and a cell of standard sensitivity (length, 12·5 cm; internal volume, 31 ml) was used for experiments with the much smaller amounts of acetaldehyde evolved from suitable weights of rhamnose and polysaccharides.

Rate of aspiration of acetaldehyde—

Preliminary experiments showed that amounts of acetaldehyde of up to 1 mg were quantitatively aspirated within 1½ hours at nitrogen flow-rates of 300 to 350 ml per minute. In routine determinations, an aspiration period of 2 hours is allowed, and this time is in good agreement with that found by other investigators.[6]

Recovery of acetaldehyde after periodate oxidation of rhamnose—

A standard solution, made by dissolving 400 mg of rhamnose monohydrate in 500 ml of water, was used in preliminary experiments to establish the reaction conditions for quantitative periodate oxidation, volumes of this solution ranging from 0·50 to 5·00 ml being used. Considerable amounts of formic acid are usually released in periodate oxidations of carbohydrate materials, and the reaction medium must be adequately buffered to prevent an increase in acidity as the oxidation proceeds. The most satisfactory results were obtained when a pH of 7·0 was maintained with the phosphate buffer system described above. For individual weights

of rhamnose monohydrate in the range 1·5 to 6·0 mg, weights of acetaldehyde corresponding to recoveries of rhamnose monohydrate of between 96 and 104 per cent. were obtained by the procedure given below. Similar recoveries were obtained for fucose and for rhamnose, to which was added equal weights of arabinose, galactose and glucuronic acid.

Method

The apparatus and reagents are as described under "Experimental."

Procedure—

Transfer the standard rhamnose solution, or the weighed sample of rhamnose, to the three-necked flask *via* neck A (see Fig. 1). Add 1 ml of potassium arsenite solution, 5 ml of phosphate buffer solution and sufficient water to make the final volume up to 17 ml. Place in the dropping funnel, by pipette, 3 ml of sodium *m*-periodate solution. Assemble the apparatus as shown in Fig. 1, immersing the pre-trap, P, in an acetone - solid carbon dioxide mixture, and trap T in a flask of liquid nitrogen. Start the reaction by opening tap B, simultaneously inserting the nitrogen-delivery tube into socket C. Maintain a nitrogen flow-rate of 300 to 350 ml per minute for 2 hours. During this time a considerable amount of ice is deposited in pre-trap P, but there is no retention of acetaldehyde, which is collected in trap T. The wide-bore entry shown for pre-trap P is necessary to prevent the deposition of ice from blocking the flow system.

When the reaction is completed, transfer the acetaldehyde quantitatively[24] from trap T to a gas-cell of suitable sensitivity, previously calibrated (under standardised instrumental conditions) with known weights of acetaldehyde.

Spectroscopic conditions for calibration

Under the conditions described above, infrared spectroscopy showed that formaldehyde and formic acid were not present in admixture with the acetaldehyde. The strong carbonyl absorption by acetaldehyde vapour at 1750 cm^{-1} can therefore be used for calibration purposes. In unusual reactions, however, other carbonyl compounds could conceivably be trapped together with the acetaldehyde; under such circumstances (which should always be tested for) the choice for calibration purposes of suitable individually separated peaks in the infrared absorption spectrum of each of the components present in the mixture will permit their individual concentrations to be determined simultaneously.[27]

Traces of carbon dioxide and water vapour will not interfere with normal determinations of acetaldehyde; the function of the pre-trap, P, is to reduce the water vapour collected in trap T to a minimum, so that the sodium chloride windows usually fitted to gas-cells will not become fogged more quickly than can be avoided, with resulting invalidation of the calibration curve being used. Recently, we have used windows of "Irtran-4" glass for our infrared cells, and traces of water vapour are then entirely without effect, and calibration curves remain valid much longer. The sole disadvantage is the high cost of this type of optical glass.

The effect of adding alanine to the reaction mixture

In the method described by Nicolet and Shinn,[10] the addition of 200 mg of alanine to all reaction mixtures was recommended, presumably to react with any formaldehyde produced and thus eliminate the possibility of its being aspirated together with the acetaldehyde. Under the reaction conditions given above, the volatilisation of formaldehyde does not occur, and the addition of alanine is not necessary. The effect of the addition of alanine was, however, investigated, and it was found, unexpectedly, that the addition introduces a source of error that leads to high results. Nicolet and Shinn[10] reported that alanine undergoes reaction with

periodate at a rate which is one-thousandth of that of the hydroxy amino-acids, serine and threonine. Other authors have reported[28] that the oxidation of alanine with periodate at room temperature is negligible at pH 7·6, the optimum pH being 8·0.

In experiments with threonine and alanine (micro-analytical reagent grade) under the reaction conditions given above, it was found that threonine is quantitatively oxidised to acetaldehyde. In contrast, alanine undergoes slow oxidation, resulting in the production of 0·30 mg of acetaldehyde (average of five determinations) from 200-mg samples of alanine in 2 hours. A similar result was obtained with two other specimens of alanine from other sources. (Although an aspiration period of 2 hours was allowed, the effective duration of the periodate-oxidation reaction, which is arsenite controlled, is much less.)

The implication of these results is that, if 200 mg of alanine are added to 1·64 mg of rhamnose, the recovery of acetaldehyde after aspiration for 2 hours will be 0·44 mg (from the rhamnose) *plus* 0·30 mg (from the alanine). The addition of alanine should therefore not be made, since high recoveries will be obtained.

The hydrolysis stage for polysaccharides before periodate oxidation

Polysaccharides containing rhamnose must be completely hydrolysed, so that all the rhamnose is available in the free form for the periodate-reaction stage. The hydrolysis conditions required by two gum exudates, differing widely in their rhamnose content, were investigated; the samples used had been carefully purified by electrodialysis, since it is known[29] that traces of heavy-metal ions can cause decomposition of acetaldehyde. It was found that hydrolysis under reflux in N sulphuric acid and in 2 N sulphuric acid followed first-order kinetics; the times required for complete hydrolysis were 6 hours and 2½ hours, respectively. The decomposition of rhamnose was studied under these conditions, and although no detectable decomposition occurred with either system in the times stated, the hydrolysis with 2 N acid for 2½ hours was preferred for the *Acacia* specimens. For different polysaccharide materials, however, the hydrolysis conditions required would have to be established before the remainder of the analytical procedure can be carried out.

After the hydrolysis stage, the strongly acidic hydrolysate must be neutralised before the start of the periodate oxidation, and the procedure described below is proposed.

Hydrolysis with 2 N sulphuric acid is carried out in a 50-ml flask fitted with a short water-condenser, the flask being heated in a water-bath at 100° C for 2½ hours. A suitable weight of the polysaccharide (approximately 200 mg for a rhamnose content of 1 per cent., and approximately 50 mg for a rhamnose content of 10 per cent.) is placed in the flask, and dissolved in 12 ml of 2 N sulphuric acid before the heating is started. After it has been heated for 2½ hours at 100° C, the hydrolysate is transferred with careful rinsing to a 25-ml calibrated flask. The hydrolysate is then made neutral to methyl orange indicator by the addition of the required volume of 5 N sodium hydroxide, the volume being finally adjusted to 25 ml with water. Portions (3, 4 or 5 ml) are then placed in the three-necked reaction flask (Fig. 1) for periodate oxidation. (If desired, smaller samples of polysaccharide, *e.g.*, 5 to 10 mg for a rhamnose content of 10 per cent., can be hydrolysed, neutralised and transferred directly to the three-necked flask for single determinations.)

When the amount of rhamnose present is extremely small, however, a useful technique is to transfer a known weight of rhamnose to the periodate-oxidation flask with the portion of polysaccharide hydrolysate. The rhamnose content of the polysaccharide can then be calculated from the apparent excess in the recovery of the added rhamnose. This technique allows the spectroscopic measurement to be made with respect to the most sensitive portion of a calibration curve, and its use has already been described in greater detail.[30]

Results

Determinations on samples of *A. senegal* (gum arabic) gave a rhamnose content of 13·0 per cent., a value in excellent agreement with that commonly accepted for this polysaccharide. Determinations on the exudate from *A. karroo* gave a rhamnose content of 2·05 per cent., a result that substantiates the provisional value reported by Charlson, Nunn and Stephen,[9] who found this amount "too small to estimate with accuracy." Results obtained by the proposed method for some other *Acacia* specimens, hitherto unstudied, are given in Table I; the replicates indicate the confidence that can be shown in determinations of extremely low rhamnose contents.

Table I

The rhamnose content of some *Acacia* species

Acacia species	Weight of gum sample in portion taken, mg	Weight of rhamnose added (*a*), mg	Weight of acetaldehyde found (*b*), mg	Total recovery of rhamnose ($c = b \times 164/44$), mg	Weight of rhamnose in gum sample ($c - a$), mg	Rhamnose present, %
A. nilotica	40·4	0·38	0·148	0·55	0·17	0·4
	40·4	0·38	0·145	0·54	0·16	0·4
A. arabica	41·6	0·38	0·148	0·55	0·17	0·4
	41·6	0·38	0·150	0·56	0·18	0·4
A. nubica	40·0	0·38	0·166	0·62	0·24	0·6
	40·0	0·38	0·169	0·63	0·25	0·6
A. drepanolobium ..	48·3	Nil	0·129	0·48	0·48	1·0
	48·3	Nil	0·137	0·51	0·51	1·1
A. campylacantha ..	12·5	Nil	0·236	0·88	0·88	7·0
	12·5	Nil	0·231	0·86	0·86	6·9

Conclusions

The method described has proved to be straightforward and has given satisfactory results in routine use in this laboratory during the past 6 months. Although it has been used specifically to investigate samples of gum exudates, it is clear that the method is of general application for methyl pentose determinations in the polysaccharide group, provided that the hydrolysis conditions required are established for each material under examination. The method gives an over-all accuracy greater than has been available to date; further, the method gives reliable results at lower levels of rhamnose than could previously be determined.

The procedure described gives quantitative recovery of threonine, and the method outlined should facilitate determinations of this amino-acid in proteins. Other analytical reactions that can be based on a conversion to acetaldehyde include the determination of lactic acid,[15,20,31] and that of alanine after reaction with ninhydrin.[32]

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The Use of "Biogel-P" in the Gel Filtration of Polysaccharides

By D. M. W. ANDERSON, I. C. M. DEA, S. RAHMAN, and J. F. STODDART

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LATHE and RUTHVEN[1] suggested that columns of swollen starch might provide a simple chromatographic method for determining the molecular size of proteins and polysaccharides. Gel filtration on cross-linked dextrans ("Sephadex") has, however, been much more successful, particularly for proteins,[2–5] and molecular weights of up to 225,000 can now be determined.[6] Cross-linked polyacrylamide gels have been used for gel-filtration studies on proteins.[7] These are now available commercially ("Bio-Gel P") and they offer the opportunity of studying the gel-filtration behaviour of polysaccharides on noncarbohydrate material.

Accordingly, we have examined different grades of "Biogel-P" for their possible application to the estimation of the molecular size of polysaccharides. For "Bio-Gel P 300" the empirical relationship between log $\overline{M}_n$ and elution volume[2,8] is linear for values of $\overline{M}_n$ between 5000 and 125,000; although the useful working range extends slightly beyond these values (cf. ref. 9) the exclusion limit of "Biogel-P 300" for polysaccharides appears to fall considerably below the value of 300,000 quoted commercially and found, presumably, for proteins. We have used columns measuring 2·5 × 50 cm. and 5·0 × 50 cm. at a loading of 2—10 mg. of polysaccharide. Calibration can be effected with dextran fractions having known values of $\overline{M}_n$ and, when molar sodium chloride is used as eluant, we have found such a calibration to be valid for acidic polysaccharides. Thus two gum fractions (obtained[10] from *Acacia senegal* gum), for which osmotic-pressure measurement had indicated $\overline{M}_n$ 105,000 and 37,000, respectively, gave $\overline{M}_n$ = 99,000 ± 10,000 and $\overline{M}_n$ = 35,000 ± 3,000 by gel filtration. A sample of the degraded (autohydrolysis) gum, having $\overline{M}_n$ = 4400 (periodate end-group analysis, as formaldehyde) gave $\overline{M}_n$ = 4800 ± 500 by gel filtration. The elution pattern of a de-ionised sample of the whole gum indicated that the molecular-weight distribution extended over a very wide range.

More experiments with "Bio-Gel P" materials are necessary to assess the importance of their application in fractionation and degradative studies and to establish their validity, applicability, and useful working range for molecular-weight estimations of polysaccharides.

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SOME OBSERVATIONS ON MOLECULAR WEIGHT ESTIMATIONS BY MOLECULAR-SIEVE CHROMATOGRAPHY

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Use of the so-called "gel filtration" technique for separating substances on a molecular size basis is increasing rapidly. "Gel filtration" columns can also be calibrated for direct molecular weight estimations of polystyrenes[1], proteins[2–6], peptides[7], and polysaccharides[8] if suitable molecular-sieves and valid standards of known molecular weight are available.

Unfortunately, several sources of confusion exist in the literature. Many of the papers concerned have been published in either remote or applied biological journals; we choose to draw attention to these in an international analytical journal in the hope that increased fundamental interest in some of the present sources of difficulty will result.

The validity of PORATH AND FLODIN's term[9] "gel filtration" as a suitable description of the essential process(es) involved has been questioned legitimately[10–14]. The discussion regarding the relative merits of the alternatives "exclusion chromatography", "restricted diffusion chromatography", and "gel permeation chromatography" has been reviewed by DETERMANN[15], who proposed a differentiation between "gel filtration" and "gel chromatography". The term "molecular-sieve filtration" has also been used[16].

ACKERS[17] suggested that "molecular exclusion" and "restricted diffusion" are distinguishable primary molecular-sieving effects, the relative importance of which varies with the type of molecular-sieve used. Almost simultaneously, and in direct contrast, LAURENT AND KILLANDER[10] concluded that the "decreased diffusion rate" was not a contributory process on the basis of the insensitivity of elution position to rate of flow. Until this disagreement is resolved—and, moreover, since gel-formers are not the only type of bed materials that can give the essential molecular-sieving effect[11,18,19]—it appears that none of the terms referred to above is generally acceptable. Although TISELIUS, PORATH AND ALBERTSSON[20] stated (*cf.* ref. 15) that the technique "differs basically from common chromatography", some of their supplementary remarks support the view that the essential process is described more appropriately as "chromatography" than as "filtration". Assuming, however, that the term "molecular-sieve" is accepted to have the physico-chemical significance required to describe those porous column-packing materials which allow large molecules to be eluted faster than small molecules, the name "molecular-sieve chromatography" (M-SC)[21] appears to be the most appropriate of those suggested to date. Perhaps it is not yet too late for the adoption of this term in preference to "gel filtration".

Confusion also arises at present from the lack of agreement regarding the best method of relating elution volumes to molecular weights. IWATSUBO AND CURDEL[22] plotted elution volumes directly against molecular weight, and obtained a smooth curve. In order to obtain linear relationships, several authors[2,3,5,23,24] have each elected to plot some different function of the elution volume against the logarithm of the molecular weight, whilst others have related the cube root of the "distribution coefficient", K_d, linearly to the square root of the molecular weight[26]. In contrast, SANFELIPPO AND SURAK[27] observed a linear relationship between K_d and the *reciprocal* of the logarithm of the molecular weight for hormonally-active proteins and peptides on cross-linked dextran gels.

Defining K_d as the fraction of the internal volume, V_i, that is accessible to the solute, then

$$K_d = \frac{V_e - V_0}{V_i} \tag{1}$$

where V_0 is the void volume of the column and V_e is the elution volume[28]. ACKERS[17] has interpreted "molecular-sieve" processes in terms of a restricted diffusion mechanism in which he relates K_d to the Stokes' radius, a, of a macromolecule that is diffusing within a restrictive barrier of effective pore radius, r, by the RENKIN[29] equation

$$K_d = (1 - a/r)^2 \left[1 - 2.104\, a/r + 2.09\, (a/r)^3 - 0.95\, (a/r)^5\right] \tag{2}$$

From this, ACKERS computed theoretical values for K_d for known values of a/r; plotting these values of K_d *directly* against a/r gave good agreement with the experimental values. By plotting all ACKERS' computed values of K_d against the logarithm of a/r, we obtain curve A in Fig. 1.

An alternative approach follows from PORATH's theory[26]; on the assumption

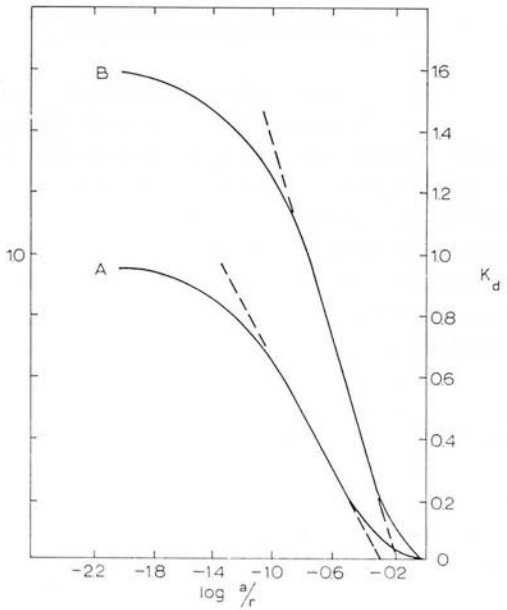

Fig. 1. Plot of K_d *versus* log a/r. Curve A: from ACKERS' theory; curve B: from PORATH's theory.

that gel pores are conical in shape, PORATH derived, for the exclusion process, a theoretical formula which, in terms of the symbols already introduced here, is equivalent to

$$K_d = k(1 - a/r)^3 \quad (3)$$

where k is a constant, equal to 1.64 for dextran fractions on "Sephadex" gels. For this value of k, we have calculated values of K_d for known values of a/r; a plot of K_d against $\log a/r$ gives curve B in Fig. 1.

These theoretical treatments can be used in a qualitative way to account for the empirical correlations of other investigators. For either curve A or B in Fig. 1, any restricted central portion that can be considered essentially linear is of the general form

$$K_d = -k_1 \log a/r - k_2 \quad (4)$$

where k_1 and k_2 are constants. Since a is proportional to some fractional power of the molecular weight M, eqn. (4) can be expressed as

$$K_d = -\mathrm{b} \log M + \mathrm{c} \quad (5)$$

where b and c are constants. A plot of K_d against $\log M$ is therefore linear[24] over a restricted range which is dependent on the nature of the solute molecules and on the type of molecular-sieve. Departures from linearity[2,5] for solutes which can penetrate the molecular-sieve pores to only a very small extent are predicted by either curve A or B in Fig. 1.

Since V_0 and V_i are constants for a particular column, eqn. (1) can be re-written as

$$V_e = -\mathrm{b}' \log M + \mathrm{c}' \quad (6)$$

where b' and c' are constants. This form of plot, used by ANDREWS[2], is the simplest possible correlation between the elution volume and $\log M$; we have found it the most convenient and reliable plot for routine use. The introduction of other constants, such as V_0[5], may facilitate comparison of solute behaviour on dimensionally different columns of the same gel-former, but this is not necessary for calibration purposes.

Almost all the other logarithmic forms of plot to which reference has been made[2,3,5,23,30] may be regarded as variants (merely involving changes in the associated constants) of eqn. (5). The equation

$$\log M = -\mathrm{a}(V_e/V_0 - 1) + \mathrm{b} \quad (7)$$

published (with an incorrect sign) by WHITAKER[5], and subsequently used correctly by LEACH AND O'SHEA[3], appears to be needlessly complex. The relationship of SANFELIPPO AND SURAK[27] is exceptional in that it does not follow directly from eqn. (5).

Values of K_d, as defined by eqn. (1), are dependent on the values found for V_i, and there is disagreement regarding how this can best be determined experimentally. Values for V_i have been determined (*a*) from expressions using the water regain of the gel[1,6,23–25], and (*b*) from the difference between the elution volumes of tritiated water and some substance that is completely excluded from the molecular-sieve[17]. Such determinations of V_i include, with the internal volume, the water of hydration which is associated with the gel and is generally inaccessible to polymer solute molecules[28].

In addition to the poor theoretical justification for determining V_i from an expression using the water regain, there are experimental difficulties; the internal volume of a gel packed in a column may differ from that determined experimentally under centrifugal force[24].

It is therefore suggested that K_d be defined according to the practice introduced by ANDREWS[2]; V_i is defined as the difference between the elution volume at which molecular-sieving ceases to be operative for small molecules (of the polymer system under test) and the void volume, V_0, of the column. This point becomes more important in the light of a recent theoretical treatment of "Sephadex gel filtration" by SQUIRE[31], who claims that his equations might apply throughout the entire range of molecular weights, even for very small molecules. The molecular-sieve process breaks down for relatively small molecules; on a "Bio-Gel P300" column, capable of molecular weight estimations of polysaccharides[8] in the range 5,000–125,000, the elution volumes of glucose and sucrose are identical. On ANDREWS' basis[2] this elution volume equals V_0+V_i, and K_d is therefore unity for both glucose and sucrose. The introduction of constant k into eqn. (3) is necessary to account for the experimental observation[1,2,26] that, in a linear plot of $K_d^{\frac{1}{3}}$ against $M^{\frac{1}{3}}$, the *intercept* on the $K_d^{\frac{1}{3}}$ axis is greater than unity. This fact was not realised, apparently, in earlier papers[6,15] in which the straight line drawn through a number of observations would have represented the experimental data better had it not been made to pass through unit value for $K_d^{\frac{1}{3}}$ at $M^{\frac{1}{3}}=0$.

LAURENT AND KILLANDER[10] have pointed out that PORATH's assumption[26] that a is directly proportional to $M^{\frac{1}{3}}$ is true for flexible macromolecules only[32]. In contrast, SQUIRE[31] assumed a to be directly proportional to $M^{\frac{1}{3}}$, and he deduced a linear relationship between $(V_e/V_0)^{\frac{1}{3}}$ and $M^{\frac{1}{3}}$ for proteins and dextran fractions. Both PORATH and SQUIRE claim good agreement with the experimental data, although CARNEGIE has found that SQUIRE's relationship is not as satisfactory as PORATH's for peptides on columns of "Sephadex G25" with phenol–acetic acid–water (1:1:1, w/v/v) as solvent. At present, however, practical tests of such theories are not very sensitive (*cf.* ref. 10). In contrast, the relationship between K_d and log M has the merit of holding regardless of the value of the fractional power to which M has to be raised so as to be proportional to a, provided that the fractional power is constant for the polymer series under investigation.

ALBERTSSON[33] has shown that the BRÖNSTED relationship[34] between the partition coefficient and the molecular weight of globular proteins applies to their distribution in certain two-phase aqueous systems. If such a relationship holds in molecular-sieve chromatography, log K_d would be expected to be proportional to $M^{\frac{2}{3}}$ (*cf.* ref. 21) if the temperature is constant.

Thin-layer chromatography of proteins on "Sephadex" has also been used for molecular weight estimations[2,35], and this technique has some features in common with molecular-sieve chromatography. Thus eqn. (1) can be written

$$K_d = \frac{V_e - V_0}{V_i' - V_0} \tag{8}$$

where

$$V_i' = V_0 + V_i \tag{9}$$

Now the elution volume for a solute molecule is inversely proportional to the distance

moved by that molecule down a column in a given time. Comparing the migration distances on a thin-layer chromatogram with those on a column, eqn. (8) may be re-written in the form

$$K_d = \frac{(d_0 - d_e)d_i'}{(d_0 - d_i')d_e} \quad (10)$$

where d_0, d_e and d_i' are the migration distances corresponding to the elution volumes V_0, V_e and V_i' respectively. This relationship is similar to that used by CARNEGIE[7] in his peptide mapping technique. Since $d_i'/(d_0 - d_i')$ is a constant, it follows from eqn. (10) that

$$K_d = c_1(d_0/d_e - 1) \quad (11)$$

where c_1 is a constant. If the R_0 value be defined by d_e/d_0, then

$$K_d = c_1(1/R_0 - 1) \quad (12)$$

so that a plot of K_d against the reciprocal of R_0 should be linear. In contrast, MORRIS[35] has found that a plot of K_d against R_{Hb} (which corresponds directly to R_0) is linear for proteins on "Sephadex" G100 and G200, and CARNEGIE[7] found an approximately linear relationship between d_e and log M for peptides on "Sephadex" G25.

These comparatively new analytical techniques for estimating molecular weights are clearly in their infancy. The potential applications are so wide that it is important for the fundamental difficulties referred to here to be resolved, if progress is to be made on other than an empirical basis.

We thank Professor Sir EDMUND HIRST for his interest in this work, Dr. J. H. KNOX for helpful discussion on some aspects of chromatography, and the Science Research Council for a maintenance grant (to J.F.S.).

SUMMARY

A critical review is given of theoretical aspects of the quantitative application of molecular-sieve chromatography to the estimation of molecular weights of macromolecules. Particular reference is made to some of the inconsistencies, controversies, and sources of confusion which exist in the literature at present.

RÉSUMÉ

Une revue critique est présentée sur les aspects théoriques de l'application quantitative de la chromatographie sur colonne avec remplissage de tamis moléculaire, en vue d'une estimation de poids moléculaires. Les auteurs se sont référés spécialement à quelques controverses et sources de confusion existant actuellement dans la littérature.

ZUSAMMENFASSUNG

Es wird ein kritischer Überblick gegeben über theoretische Aspekte der quantitativen Anwendung der Molekularsieb-Chromatographie auf die Abschätzung von Molekulargewichten von Makromolekülen. Besonders werden einige kontroversen und Unstimmigkeiten in der gegenwärtigen Literatur berücksichtigt.

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Separations of Sugars on "Chromagrams"

Recently, "Chromagram" sheets for thin-layer chromatography have become available commercially (Messrs. Kodak Ltd., Kirkby, Liverpool). We have examined the polycarbonate and silica-gel forms of these, and have obtained very rapid, good separations of many sugars, without the tedium of plate preparation. The uniformity of coating allows reproducible R_F values to be obtained without difficulty; moreover, the solvent front runs evenly, and the absence of "edge-effect" facilitates comparison of R_F values of unknown sugars with those of standards on the same chromagram.

EXPERIMENTAL

Chromagrams were developed by the ascending technique in a small tank (Shandon, for thin-layer chromatography) lined with filter sheet to assist vapour-phase equilibration.

The polycarbonate coating was pre-treated by immersion in phosphate buffer (0.2 M, pH 6.8). After the chromagram had been air-dried, 0.5 μl of a 5% aqueous solution of sample was applied. The solvent system, propan-1-ol–ethyl acetate–water (10:3:1), recommended by Messrs. Kodak, Harrow Division, was used; development for 5 h was required. The chromagrams were sprayed with (*a*) saturated ethanolic aniline oxalate (followed by heating in an oven at 140° for 2–3 min), to detect reducing sugars, or (*b*) an aqueous solution of periodate and alkaline permanganate[1] to detect sugar alcohols. Table I gives the R_F values obtained.

TABLE I

R_F VALUES OF SOME SUGARS ON POLYCARBONATE (0.2 M PHOSPHATE BUFFER, pH 6.8) CHROMAGRAMS USING PROPAN-1-OL–ETHYL ACETATE–WATER (10:3:1)

D-Galactose	0.13	D-Xylose	0.44
D-Mannose	0.31	D-Ribose	0.49
D-Glucose	0.18	L-Arabinose	0.29
L-Rhamnose	0.58	Maltose	0.10
L-Fucose	0.49	Lactose	0.05
D-Glucuronic acid	0.03	D-Arabinitol	0.37
D-Galacturonic acid	0.03	Erythritol	0.48
D-Fructose	0.28		

The behaviour of some sugars on the silica-gel chromagrams, pre-treated with 0.1 N boric acid, was examined in two different solvent-systems[2]: solvent 1, butan-1-ol–acetone–water (4:5:1); solvent 2, butanone–acetic acid–water (3:1:1). For these solvents, development of 10 cm required only 1 h. The chromagrams were sprayed with the same reagents as before. The R_F values observed are given in Table II.

TABLE II

R_F VALUES OF SOME SUGARS ON SILICA-GEL (0.1 N BORIC ACID) CHROMAGRAMS

	Solvent 1[a]	*Solvent 2*[a]
D-Galactose	0.32	0.36
D-Mannose	0.43	0.46
D-Glucose	0.41	0.41
L-Rhamnose	0.61	0.58
L-Fucose	0.50	0.48
D-Glucuronic acid	0.05	0.34
D-Galacturonic acid	0.06	0.42
D-Fructose	0.31	0.43
D-Xylose	0.47	0.54
D-Ribose	0.45	0.57
L-Arabinose	0.43	0.48
Maltose	0.29	0.30
Lactose	0.20	0.25
Galactitol	0.14	0.43
D-Arabinitol	0.28	0.47
Erythritol	0.44	0.52
Glycerol	0.49	0.56

[a]Solvent 1: Butan-1-ol–acetone–water (4:5:1). Solvent 2: Butanone–acetic acid–water (3:1:1).

The speed and ease with which good separations of sugars can be achieved, especially on the silica-gel chromagrams, make these analytical aids attractive to the carbohydrate investigator.

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STUDIES ON URONIC ACID MATERIALS

PART XV*. THE USE OF MOLECULAR-SIEVE CHROMATOGRAPHY IN STUDIES ON *Acacia senegal* GUM (GUM ARABIC)

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INTRODUCTION

Lathe and Ruthven[2], and Andrews and Roberts[3], have suggested the possibility of applying molecular-sieve chromatography (gel "filtration") to molecular-weight estimations on polysaccharides. Some of the experiments described in this paper on the application of this technique to *Acacia senegal* gum have been reported in a preliminary communication[4].

MATERIALS AND METHODS

The nodules of *A. senegal* (syn. *verek*) were collected by (the late) Mr. M. P. Vidal-Hall, Gum Research Officer, Republic of the Sudan, at Qala en Nahal, Kassala Province, as the first collection of the 1960 gum season.

Nitrogen, ash, and moisture determinations

Nitrogen was determined by a semi-micro Kjeldahl method, moisture by heating to constant weight at 105°, and ash by heating (muffle furnace) to constant weight at 550°.

Viscosity measurements

Determinations were made in M sodium chloride, in a suspended-level, dilution viscometer at 25° (flow time for M sodium chloride, 189.9 sec).

Uronic acid determinations

Uronic acid content was determined by a vapour-phase, i.r. method after decarboxylation with hydriodic acid[5].

Methoxyl determinations

A vapour-phase, i.r. method was used[5].

*For Part XIV see ref. 1.

Polysaccharide hydrolyses

Polysaccharides were hydrolysed with N sulphuric acid for 7 h at 100°. These conditions do not cause any extensive hydrolysis of the uronic acid linkages in *A. senegal* gum; this was taken into account when determining proportions of galactose. Hydrolysates were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+ form) and concentrated at *ca.* 35° on a rotary evaporator.

Sugar ratios

These were determined by chromatographic separation on Whatman 3MM paper, followed by elution and colorimetric estimation by the phenol–sulphuric acid method[6]. After periodate oxidation, rhamnose was also determined, as acetaldehyde, by a vapour-phase, i.r. method[7]. Sugar compositions were calculated as anhydro-sugar residues.

Periodate oxidations

Unless otherwise stated, these were carried out at room temperature in darkness using excess of sodium metaperiodate. Formic acid was estimated potentiometrically[8]. Formaldehyde was estimated colorimetrically with chromotropic acid[9].

Paper chromatography of sugars

Whatman No. 1 and 3MM papers were used with the following solvent systems (v/v): (*a*) benzene–butan-1-ol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*c*) butan-1-ol–ethanol–water (4:1:5, upper layer); (*d*) butan-1-ol–acetic acid–water (4:1:5, upper layer); (*e*) ethyl acetate–pyridine–water (10:4:3); (*f*) butanone–acetic acid–water (9:1:1, saturated with boric acid). Chromatograms were developed with aniline oxalate, *p*-anisidine hydrochloride, alkaline silver nitrate, or the periodate–permanganate reagent.

Thin-layer chromatography of sugars

This was carried out on "Chromagram" sheets (Kodak Ltd., Kirkby, Liverpool) of polycarbonate or silica gel[10], using the following solvent systems (v/v): (*g*) propan-1-ol–ethyl acetate–water (10:3:1) with polycarbonate sheets; and (*h*) butan-1-ol–acetone–water (4:5:1), or (*i*) butanone–acetic acid–water (3:1:1) with silica-gel sheets.

Molecular-sieve chromatography

This was carried out on columns (6.0 × 50 cm) of "Bio-Gel P300" (Bio-Rad Laboratories, Richmond, California) using M sodium chloride as eluant[4]. To prevent deformation by "wall effects", columns were pre-treated with 1% dichlorodimethylsilane in benzene at 60°. After oven-drying, columns were packed with gel that had been allowed to swell in M sodium chloride for 2 days. The gel slurry was added continuously to the column; a thin layer of glass beads supported the gel and kept the "dead space" to a minimum. To stabilise the soft top-surface of the P300 gel, 1-cm

layers of "Bio-Gel P200" and "Bio-Gel P10" were applied successively to the column. Eluant was allowed to flow for 2 days before the columns were calibrated with dextran fractions (Pharmacia Ltd., Uppsala) of known, number-average, molecular weight ($\overline{M}_n$). Polysaccharide (*ca.* 10 mg), dissolved in 1.5M sodium chloride (1 ml), was applied to the column by careful layering beneath the M sodium chloride. Fractions, collected from a 2-ml siphon by an automatic collector, were screened by the phenol–sulphuric acid method[6]. Elution volumes (V_e) were estimated to the nearest ml from peak maxima.

RESULTS

Fractional precipitation of A. senegal *gum with sodium sulphate*

The gum (40 g) was dissolved in water (800 ml), filtered, and electrodialysed. Analyses on the freeze-dried product are shown in Table I.

A solution of the purified gum (25.4 g) in water (500 ml) was maintained at 28°. Anhydrous sodium sulphate was added in small portions with constant stirring. Precipitation commenced at concentrations approaching 40% (w/v); at 40%, a pale-brown material rose to the surface and was removed (Fraction I). Two further fractions, II and III, much lighter in colour, were obtained on continued, slow, stepwise addition of very small portions of sodium sulphate; eventually, the supernatant solution contained polysaccharide material which was not precipitated from a saturated solution of sodium sulphate, and this yielded Fraction IV. The fractions were dialysed against tap water until free of sulphate and were then electrodialysed to ensure complete removal of inorganic ions. Analytical data for the freeze-dried fractions are given in Table I.

Autchydrolysis of A. senegal *gum*

A sample (4 g) of electrodialysed gum was dissolved in water to give a 2% solution (pH, 2.8). Autohydrolysis on a boiling water-bath was followed polarimetrically[11]. After 50 h, the solution was cooled, filtered (to remove denatured protein), and dialysed against water (3 × 2 l). Dialysis was completed against running tap-water, and freeze-drying gave the degraded gum (2 g), $[\alpha]_D$ −11° (*c* 1.0, water) (Found: moisture, 9.7; uronic acid, 19.2; galactose, 68; arabinose, 2%). Hydrolysis of the degraded gum indicated the presence of two aldobiouronic acids, which had R_{Gal} values of 0.22 (major component) and 0.59 (minor component) in solvent (*b*), and were chromatographically identical with 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, respectively. Chromatographic examination of the diffusate from the degraded gum showed it to contain galactose, arabinose, rhamnose, three (major) neutral disaccharides, traces of the two aldobiouronic acids, and oligosaccharide material. Further hydrolysis of a portion of the diffusate yielded more of the same aldobiouronic acids found for the degraded gum.

TABLE I

ANALYTICAL DATA FOR ELECTRODIALYSED *A. senegal* GUM AND FRACTIONS OBTAINED BY PRECIPITATION WITH SODIUM SULPHATE

	A. senegal gum	*Fractions*			
		I	*II*	*III*	*IV*
Yield, %	—	23.6	29.5	33.7	1.2
Moisture, %	11.0	14.7	7.3	8.6	—
Ash, %	0.01	0.01	0.02	0.01	—
N, %	0.33	1.01	0.12	0.02	—
Protein, % [N % × 6.25]	2.1	6.3	0.75	0.13	—
$[\eta]$, cm^3g^{-1}	20.0	33.5	14.8	10.8	—
Rhamnose, %[a]	12(14)	10(13)	12(13)	12(13)	—
Arabinose, %[a]	25(28)	24(30)	26(28)	23(25)	—
Galactose, %[a]	34(39)	29(37)	37(40)	40(44)	—
Uronic acid, %[a,b]	16.7(19)	16.0(20)	17.5(19)	16.7(18)	15.5
Methoxyl, %	0.23	0.23	0.23	0.23	0.22
$[\alpha]_D$ (*c* 1.0, water)	−31.5°	−32.7°	−32.7°	−31.5°	—
Equiv. wt.[c]	1290	—	—	—	—
Formic acid released on periodate oxidation (mole/g) × 10^3	1.58	1.56	1.59	1.60	—
Ratio of galactose/arabinose[d]	1.40	1.23	1.43	1.76	—

[a] Values in parentheses are corrected for all non-carbohydrate material.
[b] Calculated as the anhydride of glucuronic acid.
[c] By direct titration (potentiometric) with 0.02N sodium hydroxide.
[d] Calculated from the values corrected for non-carbohydrate material.

Borohydride reduction of degraded gum

Degraded gum (500 mg) was dissolved in water (100 ml), and sodium borohydride (400 mg) was added. The solution was kept for 24 h at room temperature before further sodium borohydride (100 mg) was added. After the solution had been stirred for 6 h, it was dialysed against running tap-water for 2 days. The freeze-dried product was hydrolysed to yield the same aldobiouronic acids and neutral disaccharides found in the degraded gum. In addition, paper chromatography in solvent (*f*), and t.l.c. on silica gel with solvent (*h*) indicated the presence of galactitol. No arabinitol was detected.

Periodate oxidation of degraded and reduced, degraded gum

Degraded gum did not give detectable amounts of formaldehyde on periodate oxidation. The production of formaldehyde with time from reduced, degraded gum (34.32 mg, dry wt.) was as follows: 0.25 h, 160 μg; 0.5 h, 200 μg; 1 h, 215 μg; 2 h, 220 μg; 6 h, 220 μg; 24 h, 235 μg. Assuming production of one formaldehyde molecule per average polymer unit, a value for $\overline{M}_n$ of 4,400 was calculated for the degraded gum. Taking into account its composition, this corresponds to a number-average degree of polymerisation ($\overline{P}_n$) of 27.

Controlled Smith-degradation[12] *of degraded gum*

Periodate oxidation was carried out at 2°. Degraded gum (1 g) was dissolved in water (25 ml), 50% (w/w) periodic acid (1.75 ml) was added, and the solution made up to 50 ml. After 2 days, the reaction was stopped by addition of excess of ethylene glycol. Following dialysis against running tap-water for 2 days, the solution was treated with sodium borohydride (250 mg) for 36 h. Further dialysis for 2 days was followed by hydrolysis of the acetal linkages with N sulphuric acid for 2 days at 18°. The acidic solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+ form), and concentrated on a rotary evaporator. Chromatographic examination revealed the presence of glycerol and glycolic aldehyde. Molecular-sieve chromatography on a column (2.5 × 75 cm) of "Bio-Gel P10" was used to separate such low molecular-weight materials from the Smith-degraded product (180 mg). Hydrolysis of a small portion of the latter product, with examination by paper chromatography, gave galactose, arabinose (a trace) and arabinitol [solvent (*f*)], but no galactitol or erythritol.

Molecular-sieve chromatography

Figure 1 shows the calibration plot of elution volume (V_e) against log $\overline{M}_n$ obtained with dextran fractions of known $\overline{M}_n$. For "Bio-Gel P300", this relationship[13–15] is approximately linear for values of $\overline{M}_n$ from 5,000 to 125,000; although the useful working range may extend slightly beyond these values, the exclusion limit of "Bio-Gel P300" *for the polysaccharides investigated* is apparently less than 300,000

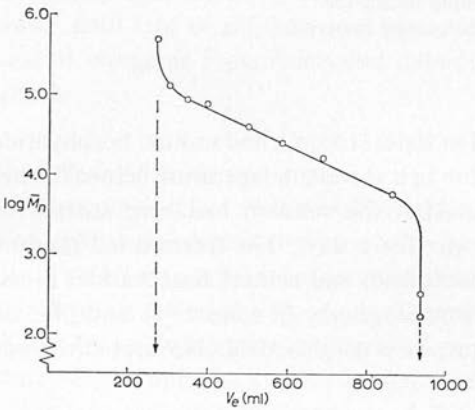

Fig. 1. Plot of elution volume (V_e) against log $\overline{M}_n$ for dextran fractions of known $\overline{M}_n$ values. ["Bio-Gel P300" column (5.0 × 50 cm), elution with M sodium chloride]. The arrows shown correspond with those on Fig. 2.

Sucrose and glucose have the same elution volume, which is defined as being equal to $V_o + V_i$, where V_o is the void volume and V_i the internal volume[14,16]. The elution volume of "blue dextran" (Pharmacia Ltd., Uppsala) was taken as the void volume, and values for the distribution coefficient (K_d) were calculated from the relationship[17], $K_d = (V_e - V_o)/V_i$. Figure 2 shows the elution patterns for *A. senegal*

gum, for the fractions (I–IV) precipitated by sodium sulphate, and for the degraded gum obtained by autohydrolysis of *A. senegal* gum. Table II gives the values found for K_d and $\overline{M}_n$; estimation of $\overline{M}_n$ for the whole gum was rendered difficult by the asymmetric nature of its elution curve.

TABLE II

ESTIMATION OF $\overline{M}_n$ BY MOLECULAR-SIEVE CHROMATOGRAPHY

	V_e	K_d	$\overline{M}_n$
A. senegal gum	(276)	—	—
Fraction I	270	0.00	—
Fraction II	294	0.04	140,000 ± 20,000
Fraction III	351	0.12	99,000 ± 10,000 (105,000)[a]
Fraction IV	532	0.40	35,000 ± 3,000 (37,000)[a]
Degraded gum[b]	884	0.92	4,800 ± 500 (4,400)[c]

[a]By osmometry; the authors thank Mr. S. Rahman for these determinations.
[b]Obtained by autohydrolysis.
[c]Periodate end-group analysis, as formaldehyde.

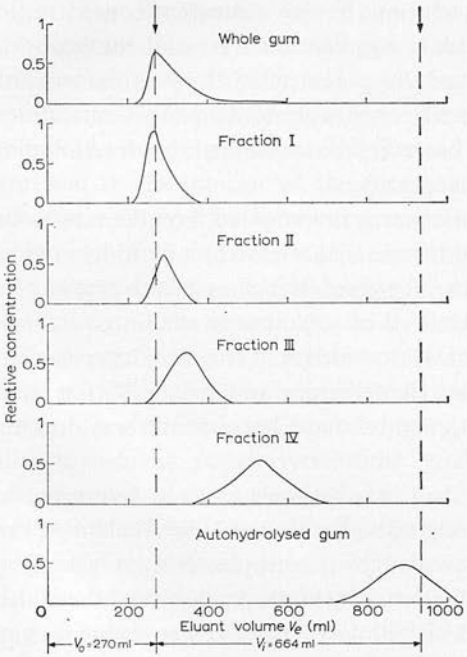

Fig. 2. Elution patterns for *A. senegal* gum, sodium sulphate fractions, and autohydrolysed gum. ["Bio-Gel P300" column (6.0 × 50 cm), elution with M sodium chloride].

DISCUSSION

Fractional precipitation of gum arabic with propan-2-ol[18] and acetone[19] has been reported. Van Beek[19] has suggested that there is a correlation between the limiting-viscosity numbers of the fractions precipitated by acetone and their content of divalent cation. In spite of the fact that metal ions were reported[20] to cause aggregation of complex acidic polysaccharides in solution, all attempts in this laboratory to repeat the experiments of Van Beek have failed.

Prolonged contact of *A. senegal* gum with organic solvents leads to insolubility difficulties, and the possibility of using salt as a fractional precipitant was therefore examined. Since the feasibility of fractional precipitation depends upon the polysaccharide in question having a broad molecular-weight distribution, *A. senegal* gum, and fractions obtained by fractional precipitation with sodium sulphate, were studied by molecular-sieve chromatography. With such fractional precipitations, the number of fractions isolated is arbitrary and is usually governed by the amount of material required for the analyses necessary to characterise the fractions. The elution patterns obtained on molecular-sieve chromatography (Fig. 2), and the limiting-viscosity numbers (Table I) of the fractions, clearly demonstrate that fractionation by "molecular" size was effected. Careful electrodialysis eliminated the possibility of traces of di- and poly-valent cations causing aggregation. Molecular-sieve chromatography and viscosity measurements were carried out in solutions having a constant concentration of univalent cations (*i.e.*, M sodium chloride). Aggregation by metal ions cannot, therefore, explain our results. Aggregation of the polysaccharide by protein is also unlikely to occur in M sodium chloride, since coacervates are broken down on addition of simple electrolytes[21]. Our results can, however, be explained by fractionation according to the molecular size of the polysaccharide.

The chemical composition of the fractions was investigated, and the results are summarised in Table I. The similar yields of formic acid released on periodate oxidation indicate that there is little variation in the degree of branching of the polysaccharides in the fractions, but the varying proportions of galactose to arabinose indicate that the gum is chemically heterogeneous. Previous evidence of chemical heterogeneity in commercial gum arabic was obtained by Heidelberger and Adams[22]; the small fraction of gum precipitated by Type II antipneumococcal horse-serum was depleted in rhamnose.

Confusion has arisen over the use of the terms *homogeneous* and *heterogeneous* in relation to plant gums and other polysaccharides. Smith and Lewis[23] claimed that the *heterogeneity* of *A. senegal* gum is revealed by electrophoresis on glass-fibre paper, whilst Jermyn[24] observed no sharp discontinuity in the properties of the molecular species after chromatography on DEAE-cellulose. *Combretum leonense* gum has been described by Aspinall and Bhavanandan[25] as *micro-heterogeneous*, *i.e.*, "a mixture of polysaccharides composed of the same structural units, which are linked in a similar manner, but are in slightly differing proportions". Norman[26] has stated that *A. senegal* gum is "not a substance of constant composition, but is con-

structed in a particular pattern from varying amounts of constituent units", and Hirst[27] has referred to it as "a mixture of closely related, molecular species". Other terms, such as *grossly heterogeneous*[25], *polydisperse*[3,28], and *polymolecular*[29], have also been employed. Unfortunately, their usage has not always been in accordance with their accepted definitions; *polydisperse* describes polymer systems containing more than one component; *polymolecular* denotes a *homogeneous* polymer having a variation in molecular weight (*cf.* ref. 20).

There is no evidence from our present investigations, nor from those of Jermyn[24], that *A. senegal* gum is *polydisperse*. On the other hand, if the gum is claimed to be *polymolecular*, the above definition of this term implies that it is a *homogeneous* polymer. The term *homogeneous* has been used[30,31] to indicate that polysaccharides are not *polydisperse*, even although, chemically, they are undoubtedly *heterogeneous*. To avoid this ambiguity, it is suggested that the term *polymolecular* be reserved for the description of those polymer systems having only a distribution in molecular weight, and the term *heteropolymolecular* be used to describe polymer systems having either a variation in monomer composition and/or a variation in the mode of linking and branching of the monomer units, in addition to a distribution in molecular weight. Defined in this way, the term *heteropolymolecular* conveys a more comprehensive description of the spectrum of related polysaccharides that comprise *A. senegal* gum.

Molecular-sieve chromatography of the degraded gum obtained on autohydrolysis (Fig. 2) indicates that $\overline{M}_n = 4{,}800$ (Table II). Degradation of the whole gum to produce units of this small size is much greater than would be expected to result from removal of labile sugar-residues (such as L-arabinofuranose and L-rhamnopyranose) from the periphery of the molecule. This observation was made by Smith and Montgomery[32], and it led them to suggest that some labile sugarresidues were present in the interior of the gum molecule. They postulated that blocks of degraded units might have been interconnected by labile residues of arabinofuranose. If this were so, it should be possible to show that some, if not all, of this arabinose is sited at the reducing end of the degraded molecules resulting from autohydrolysis. Arabinose was not, however, reported by Smith[11] to be present in the degraded gum.

This investigation shows that autohydrolysis of *A. senegal* gum results in the release of galactose residues, in addition to arabinose and rhamnose residues, with the formation of a degraded gum containing 2% of arabinose. Although autohydrolysis is sufficient to break galactopyranosidic bonds to give galactose, arabinose was not completely removed from the degraded portion that remained behind after dialysis (*cf.* ref. 33). In order to discover whether arabinose was present as the reducing end-group, the autohydrolysed, degraded gum was reduced with borohydride. The presence of galactitol, and the absence of arabinitol, in the hydrolysate of this reduced material shows that galactose occupies the reducing end-group. The 2% of arabinose in the degraded gum appears, therefore, to be sited other than at the reducing end.

Autohydrolysis is not very selective as a means of degradation. In our autohydrolysis experiments, traces of aldobiouronic acids are released, together with oligosaccharides which are small enough to pass through cellophane dialysis-tubing

(Kalle Aktiengesellschaft, Wiesbaden). Acidic material of low molecular-weight has also been obtained from autohydrolysis of gums from *A. karroo*[34] and *A. cyanophylla*[35], and this led Hirst[36] to suggest that acidic residues may occur in labile side-chains.

The methoxyl content of *A. senegal* gum has already received comment in a preliminary communication[37]. The methoxyl group is not present as the ester of D-glucuronic acid, since the methoxyl content does not decrease on treatment with N sodium hydroxide. Methoxyl groups are now known to occur commonly in plant gums in residues of 4-*O*-methyl-D-glucopyranosyluronic acid. A careful, chromatographic re-examination of the degraded gum from *A. senegal* resulted in the detection of the aldobiouronic acid, 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, the presence of which had not been recognised by earlier investigators. The sample of *A. senegal* gum used in the present study has a methoxyl content of 0.23%; this corresponds to a content of 1.4% of 4-*O*-methyl-D-glucopyranosyluronic acid. The presence of this residue in *A. senegal* gum accounts for some of the 2,3,4-tri-*O*-methyl-D-glucuronic acid obtained after hydrolysis of the methylated whole-gum[38].

Periodate oxidation of the degraded gum produces no formaldehyde, so it may be concluded, in conjunction with methylation evidence[39], that the reducing galactose residue is substituted at C-6. As a result, borohydride-reduced, degraded gum was assumed to produce one formaldehyde molecule per average unit on periodate oxidation. On this basis, a value of 4,400 for $\overline{M}_n$ was calculated for the degraded gum.

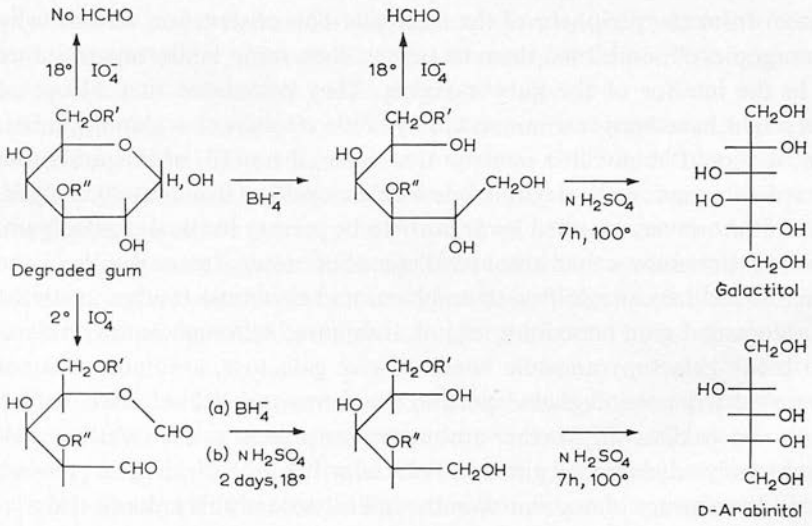

Fig. 3. Scheme of reactions carried out on autohydrolysed gum from *A. senegal*. R′ and R″ represent the remainder of the degraded gum.

The reactions carried out on the degraded gum are summarised in Fig. 3. A controlled Smith-degradation (*cf.* ref. 40) carried out at 2° provides additional

information that galactose constitutes the reducing end-group. The series of reactions proposed[32] to account for the appearance of arabinitol is evidence that the reducing galactose residues are also substituted at C-3.

If the extensive degradation of the macromolecule observed on autohydrolysis is not due to the presence of internal, labile, arabinofuranosidic bonds, certain galactopyranosidic bonds must be unusually reactive towards very mild conditions of hydrolysis, which would not normally be expected to cleave such bonds. As a result of studies on *Virgilia oroboides* gum, Stephen[41,42] has suggested that the carboxyl groups of the uronic acid residues may be responsible for "deep-seated decomposition". This fact, and the overall geometry of the *A. senegal* gum molecule, may provide the explanation for the unexpected lability of some pyranosidic bonds. More knowledge is required on the degree of branching within the molecular framework of *A. senegal* gum before a theory of more heuristic value may be advanced.

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SUMMARY

Investigations involving fractional precipitation of *A. senegal* gum by sodium sulphate lead to a discussion on the type of heterogeneity exhibited by the gum. Molecular-sieve chromatography is used to estimate number-average molecular weights. Results obtained using this chromatographic technique on the degraded gum produced on autohydrolysis indicate that such mild conditions of hydrolysis are not always very selective as a means of degradation. The degraded gum is shown to have galactose residues as reducing end-groups. There is no evidence for labile, internal, arabinofuranosyl linkages in the whole gum. In addition, chromatographic evidence is obtained for the presence of 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose residues in *A. senegal* gum.

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